DISSECTION OF NEUROMETABOLIC DISEASE USING *DROSOPHILA MELANOGASTER*: *BUBBLEGUM* AND *DOUBLE BUBBLE* MUTANTS SUGGEST NEW CELLULAR DEFECTS IN ADRENOLEUKODYSTROPHY PATHOLOGY

by

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ABSTRACT

Altered lipid metabolism is a recognized contributor to neurodegenerative (ND) conditions, both rare and common, such as Adrenoleukodystrophy (ALD) and Alzheimer's disease that affect millions of individuals worldwide. For most of these diseases, therapeutic options are limited. Significant therapeutic progress might be expected with the development of animal models. Two Drosophila mutants fail to activate long- and very long-chain FAs (acyl CoA-synthetases, ACS) were used to probe the relationship between lipid homeostasis and nervous system function with the overarching goal of determining the primary cell type affected, and the specific subcellular defect in an animal model for ALD (Figure 1.1 A, B). Adults homozygous for mutations in *bubblegum* (*bgm*) or *double bubble* (*dbb*) exhibit increased very long chain FA levels, a distinguishing feature of this human disease. The observed deficiency in activation leading to ND could be due to an inability to clear precursor and/or to generate sufficient product. Herein, we demonstrate that dietary supplementation with medium chain FA rescues ND in *dbb* mutants and partially rescues *bgm* mutants. In contrast, diet studies designed to increase the demand for product while leaving an accumulation of precursor unchanged enhance ND. Alternate approaches utilizing light/dark cycles, and blocking elongation of VLCFAs, corroborate these results demonstrating that a lack of activated FA product is causative of ND in *bgm* and *dbb*

mutants. These data provide support to the observations that therapies designed to reduce toxic accumulations in patients with ALD are ineffective and instead suggest an alternate approach to providing the missing product. Consistent with this notion is our identification of a leukodystrophy patient harboring a rare mutation in a human homolog of *bgm*. Additionally this patient harbors a mutation in a known epilepsy gene suggesting that stress, by use or by trauma, may precipitate ND in an ACS mutant background. These data provide candidates for the long-hypothesized gene-gene and gene-environment interactions proposed with ALD by identifying new genes (ACSs), mechanisms (lack of product), and environments (trauma, seizures) that contribute to disease. More broadly, these studies enhance our understanding of the required roles of lipids in the nervous system thereby contributing to knowledge of other lipid-mediated ND diseases.

This thesis is dedicated to my family for their endless love and support.

Nature composes some of her loveliest poems for the microscope and the telescope.

-Theodore Roszak, *Where the Wasteland Ends*, 1972

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CHAPTER 1

INTRODUCTION

Adrenoleukodystrophy

Leukodystrophy pathology and ALD

Leukodystrophies are a class of degenerative diseases that affect the white matter of the brain and have a broad-ranging age of onset with varying life expectancies (Gordon et al., 2014). Diagnosis is achieved by detection of abnormal white matter in the brain via MRI scans, usually after an accompanying neurological symptom warrants a visit to a clinician (issues with balance, sight, seizures, etc.). A leukodystrophy diagnosis is devastating as there are no known treatments for any form of leukodystrophy (Gordon et al., 2014; van der Knaap et al., 1999). Additionally, approximately half of individuals who present with this life-threatening clinical diagnosis do not resolve to a clear cause, underscoring the need to identify the genes responsible for leukodystrophy (Bonkowsky et al., 2010; van der Knaap et al., 1999).

Adrenoleukodystrophy (ALD), one of the most common leukodystrophies, is a rare and fatal neurodegenerative disorder with no known cure (Bonkowsky et al., 2010; Helman et al., 2015). The most common form of ALD occurs through inheritance of loss-of-function alleles of *ABCD1* on the X-chromosome (X-ALD). Therefore, the

condition usually occurs in males, most often between the ages of 5-8 (Moser et al., 2005b; Engelen et al., 2012; Kim and Kim, 2005). Females are carriers and transmit a mutated *ABCD1* allele to children, but they themselves only sometimes show a phenotype due to mosaicism in X-inactivation (Hershkovitz et al., 2002; Maier et al., 2002).

ALD occurs in approximately 1:17,000 births across all ethnicities, and inheritance of null alleles in *ABCD1* leads to incompletely penetrant and variably expressed phenotypes, making age of onset and severity of symptoms difficult to predict in males with *ABCD1* mutations (Moser et al., 2005b). Symptoms often begin with deteriorating vision, volatile behavior, hyperactivity, and decreased motor function, and progress to coma or death within a few years of diagnosis. In addition to MRI, clinicians specifically diagnose ALD by testing for an increase in very long chain fatty acids (VLCFAs) in blood and DNA sequencing of the *ABCD1* gene (Steinberg et al., 1993).

ALD phenotypes are variably expressed (see Figure 1.2 A). Forty percent of patients inheriting null alleles of *ABCD1* exhibit the most severe form of the disease (cerebral ALD) affecting the central nervous system and involving a significant inflammatory component and progressive demyelination. A second patient group (40-45%) will escape this early childhood phenotype and instead present in the second to fourth decade of life with a less severe but nonetheless debilitating form of the disease involving the peripheral nervous system (Adrenomyeloneuropathy, AMN). Approximately 10% of individuals will experience only adrenal insufficiency (Addison's Disease) and no nervous system component (Engelen et al., 2012; Steinberg et al., 1993).

ALD phenotypes are also incompletely penetrant (Figure 1.2 A). Ten percent of

individuals, harboring a null allele identical to that of an affected individual, even a family member, exhibit neither nervous system deficiencies nor adrenal gland insufficiencies. Such variation in penetrance and expressivity has led many to hypothesize the existence of environmental and genetic modifiers of ALD that if identified could assist in prediction of disease phenotype and development of therapeutic avenues (Moser, 1997; Wiesinger et al., 2015). In Chapters 2 and 3 I describe my identification of components of the VLCFA activation pathway that appear to be required for maintenance of neuronal health in both flies and humans. Our fly model of ALD is important because it provides a medium throughput platform for screens for the identification of additional genes functioning in VLCFA metabolism and in central nervous system maintenance and health.

ALD genetics

The gene responsible for X-ALD has been cloned and shown to encode a peroxisomal half ATP-binding-cassette transporter (*ABCD1*: ATP-binding cassette, sub-family D [ALD], member 1, formerly ALDP) (Mosser et al., 1993). Mutations in this gene, the only gene linked to ALD, are present in affected individuals but do not guarantee a disease phenotype (Wiesinger et al., 2015). Males pass their X-chromosome on to their daughters, and thus males hemizygous for a mutated *ABCD1* allele will transmit this allele to all of their daughters. For those heterozygous daughters, the probability of transmission of the pathogenic allele is then 50% for all offspring—males or females. *de novo* mutations in *ABCD1* have been characterized (Bezman et al., 2001; Wang et al., 2011b) and *de novo* mutation rates are estimated to be around 4%, although

the highest predictions have approached 20% (Wang et al., 2011b; Horn et al., 2013). A comprehensive list of all *ABCD1* mutations is publically available (http://www.x-ald.nl).

Very long chain fatty acids in ALD

Fatty acids are carboxylic acids with the "R" group consisting of a linear carbon chain that can very in saturation with hydrogen atoms as well as in overall chain length. The carboxylic acid can be chemically modified for various cellular purposes such as conjugation to a glycerol molecule and hydrophilic phosphate group to generate a phospholipid. In this manuscript, "very long chain fatty acids" refers to all carboxylic fatty acids with a carbon chain length of 22 carbons and greater, as well as their subsequently modified versions (such as in phospholipids).

ABCD1 encodes a peroxisomal half-transporter (Mosser et al., 1993). The current hypothesis for the mechanism of neurodegeneration in ALD states the ABCD1 protein (also called ALDP: Adrenoleukodystrophy Protein) is required for import of VLCFAs into peroxisomes where initial steps of oxidation occur: The absence of ABCD1 leads to a an accumulation of VLCFAs within tissue that is believed to be causative of cell death. This model is built on observations of accumulation of saturated VLCFAs—a well known biochemical hallmark of X-ALD (Igarashi et al., 1976). All X-ALD patients show elevated levels of VLCFAs, in particular- tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0) in the plasma, brain, and adrenal gland. There is more recent evidence for the additional accumulation of monounsaturated VLCFAs in plasma and fibroblasts of X-ALD patients (Kemp et al., 2005).

Given this association between disease state and VLCFA accumulation, the

relevance of such an increase in VLCFAs has been under investigation but has led to unclear conclusions. In multiple reports, exposure of both glial and neuronal cells to an increase in VLCFAs in culture is sufficient to induce mitochondrial dysfunction and cell death (Hein et al., 2008; Baarine et al., 2012; Mosser et al., 1993; Ronicke et al., 2009). The majority of these studies have been executed *ex vivo*, however, and it remains to be determined whether VLCFAs themselves play a central role in causing neurodegeneration in an *in vivo* model of ALD as well as whether this accumulation is necessary for neurodegeneration or simply a marker of the biochemical defect.

Despite the appeal of toxic accumulation models for disease, a few pieces of evidence do not align with the idea that neurodegeneration is solely due to an accumulation of VLCFAs. First 20% of individuals harboring disease-causing alleles of *ABCD1* while exhibiting a fully penetrant increase in VLCFAs do not display any nervous system degeneration at all (Raymond et al., 2010; Weller et al., 1992; Moser et al., 1999; Steinberg et al., 1993) (displayed in Figure 1.2 B). That these individuals do not manifest a neurodegenerative phenotype despite an accumulation of VLCFA equal to that in affected individuals suggests that VLCFA accumulation by itself is not causative of neurodegeneration. Consistent with this idea is the observation that VLCFA levels do not correlate with the degree of neurological disability (Moser, 1997). Second, animals with targeted disruption of the mouse *ABCD1* gene exhibit (like humans) an increase in VLCFAs in affected tissues without an associated central nervous system phenotype reminiscent of ALD (Forss-Petter et al., 1997; Fourcade et al., 2009; Kobayashi et al., 1997; Lu et al., 1997; Yamada et al., 2000). The most severe phenotype *ABCD1* null animals display is an incompletely penetrant AMN-like phenotype affecting the

peripheral nervous system only in aged animals (Dumser et al., 2007; Pujol et al., 2002). Third, although the sample size is low (2 patients), recipients of hematopoietic stem cell gene therapy showed a marked improvement in neurological symptoms despite plasma VLCFA concentrations remaining significantly high (Cartier et al., 2009). Taken together, these data suggest that VLCFA concentrations in ALD represent a biomarker of neurodegeneration, but have little relevance to the mechanism of neurodegeneration.

ACSs, ABCD1, VLCFAs, and the link to ALD

Acyl-CoA synthetases (ACSs), which function immediately upstream of ABC transporters in activating fatty acids and effecting their peroxisomal degradation, have also been implicated in ALD disease pathology (Hashmi et al., 1986; Lazo et al., 1988; Wanders et al., 1988a). Biochemically active acyl-CoA esters are derived from chemically inactive precursors (free fatty acids) in a two-step process, and it is the activated fatty acid that serves as substrate for the ABCD1 transporter (see Figure 1.3 A) (Kemp et al., 2011). There are four recognized ACS enzyme families: those activating short (2-3 carbons), medium (4-12 carbons), long (12-21 carbons), and very long (>22 carbons) fatty acid substrates. Although substrate specificity defines these families, it is common for specificities to overlap between families, particularly with respect to the long- and very long-chain substrates (Lu et al., 2009; Steinberg et al., 1993; Watkins, 2008; Watkins et al., 2007).

The essential role of ACSs in fatty acid metabolism is supported by their high degree of conservation in organisms ranging from bacteria to humans. Before *ABCD1* was discovered as the gene causative of X-ALD, an ACS was predicted by many to be

the gene at fault due to the marked decrease in ACS activity in patient samples (Hashmi et al., 1986; Lazo et al., 1988; Wanders et al., 1988a). It seems clear now that *ABCD1* and some ACSs require one another's presence for proper protein function. Physical interaction between ABCD1 and a cellular very long chain ACS (ACSVL1) has been demonstrated in multiple contexts. ABCD1 requires the activity of ACS esterification of fatty acids in order to transport its lipid substrate (van Roermund et al., 2008). Likewise, ACSVL1 requires ABCD1 to associate with the peroxisomal membrane and activate fatty acids for β-oxidation (Yamada et al., 1999). One study using *ABCD1*-deficient fibroblasts showed that ACSVL1 expression can, in fact, rescue the β-oxidation deficiency seen in these cells but only when it is peroxisomally-localized (as opposed to ER-localizated) implying that the subcellular localization of ACSVL1 is imperative to its function and ABCD1 may assist in the correct peroxisomal targeting (Heinzer et al., 2002). Associations between ACSVL1 and ABCD1 have also been identified independently in yeast-two hybrid screens and surface plasmon resonance (Makkar et al., 2006). Thus, as Min and Benzer inferred after their identification of neurodegeneration in *bgm* homozygotes, ACSs and ABCD1 mutations exhibit shared-loss-of-function phenotypes because they both participate in the ACSL pathway (Min and Benzer, 1999a). In Chapter 2, I report identification of a leukodystrophy patient who harbors a likely disease-causing variant of *SLC27a6,* a gene encoding an ACS in humans. Thus it appears that in humans, loss-of-function mutations in both *ABCD1* and *SLC27a6* lead to a shared loss-of-function neurodegenerative phenotype. I show in Chapter 3 that RNAi targeting the *Drosophila ABCD1* locus similarly leads to a shared neurodegenerative loss-of function phenotype with *bgm* and *dbb* mutants. Finally, I hypothesized that other

members of ACSL pathway are involved in neuronal health and maintenance. In this regard, my demonstration that *Drosophila* elongase mutants also exhibit the neurodegenerative shared loss-of-function phenotype is consistent with a requirement for ASCL pathway products in neuromaintenance.

Modifier genes in ALD

Although hundreds of *ABCD1* alleles have been described, no genotype-phenotype correlation has emerged, and inheritance of the same allele within a kindred or even shared between monozygotic twins can lead to different phenotypes (Berger et al., 1994; Korenke et al., 1996). Studies to identify modifying loci using either association/segregation studies or through gene expression analysis have had limited success due to the low number of individuals available for each study (Moser et al., 1992) (Wiesinger et al., 2015). Nonetheless, a few gene candidates are worth mentioning. Biochemically, ABCD2 and ABCD3 (also known as ALDRP and PMP70, respectively) can compensate for the decrease in B-oxidation due to the loss of ABCD1 when overexpressed in ABCD1-deficient patient fibroblasts (Netik et al., 1999). Additionally, *in vivo* overexpression of ABCD2 in ABCD1-deficient mice rescues an age-dependent AMN-like phenotype (Pujol et al., 2004). In contrast, two independent association studies attempting to link variants in ABCD2 (Maier et al., 2008) and ABCD3, and ABCD4 with prevalence or severity of ALD have yielded no correlations (Matsukawa et al., 2011). Association studies have identified two genes (HLA and SOD2) expressed in immune tissue that have been found to correlate with cerebral ALD but not with AMN or nonpenetrant ALD hemizygotes. These genes are thought to play a role in disease

severity in an individual already harboring an *ABCD1* mutation. Human leukocyte antigen (HLA) is an MHC class gene required for recognizing self vs. nonself and initiating activation of immune cells. Superoxide dismutase 2 (SOD2) is a mitochondrial protein that neutralizes damaging super oxide (O_2^-) into oxygen and H_2O_2. Superoxide is increased at sites of inflammation. It and H2O2 act as chemokines to attract and activate immune cells (Petrone et al., 1980; Kaminski et al., 2012). Therefore, SOD2 plays an important role in balancing the damaging effects of reactive oxygen with recruitment of the immune response. That the only genes that have been identified to play a role in ALD expressivity are immune-related genes speaks to the importance of the immune system in rapid progression of demyelination. Although this is an important lesson, it represents only a small step in understanding how *ABCD1* is required for neuromaintenance.

ALD animal models

Multiple attempts to generate mouse models that recapitulate central nervous system degeneration have been carried out in an effort to gain a tool to resolve the clinical heterogeneity of ALD. Similar to human disease, *ABCD1* knockout phenotypes appear to depend on genetic background. In one case, targeted disruption of mouse *ABCD1* in a C57BL/6 strain leads to the accumulation of VLCFAs in the CNS thereby recapitulating the biochemical abnormality seen in ALD patients. However, mutant mice experience a normal life span and exhibit no signs of degeneration in either the CNS or PNS (Forss-Petter et al., 1997). Knockout of *ABCD1* in a different genetic background (strain 129/Sv), however, leads to accumulation of VLCFAs and development of age-

dependent (but incompletely penetrant) AMN-type symptoms (Lu et al., 1997; Pujol et al., 2002). It is important to note that the genetic targeting strategies were very similar between these two reports as both replaced early regions of the gene (exon 1 and exon 2, respectively) with a neomycin resistance cassette resulting in an early termination gene product. Attempts at modeling ALD by genetic disruption of another gene—VLCS-1 (VLCFA acyl-CoA synthetase)—also resulted in mice with no neurological or biochemical defects possibly due to redundancy in metabolic enzymes (Heinzer et al., 2003). Although the establishment that genetic background plays a role in phenotype presentation is a valuable lesson, the utility of mouse knockout models has been hindered by the lack of a central nervous system phenotype, thus impairing the identification of genes involved in cerebral ALD.

Utilizing a fly model to study ACS function is more telling, however, as mutation of the *bubblegum* (*bgm*) gene, encoding a *Drosophila* long chain ACS (ACSL) with predicted long- and very long-chain substrate specificities results in an autosomal recessive neurodegenerative phenotype (Min and Benzer, 1999a). The *bgm* gene name refers to the appearance of a "bubbly" optic lobe in adult homozygous null mutants signifying a nervous system defect. The reported neurodegenerative abnormalities are subtle but include disorganization of the optic lobe. Neurodegeneration is associated with small differences (not reaching statistical significance) in two saturated VLCFAs (C24 and C26). *bgm* flies have, therefore, not been embraced as a strong animal model for ALD. In Chapter 2 we significantly improve the previous *Drosophila* ALD model by generating a double mutant animal harboring null alleles of *bgm* and its closest *Drosophila* homolog *double bubble* (*dbb*). We show a more completely penetrant and

more severe neurodegenerative phenotype when the functions of both *bgm* and *dbb* are disrupted. Analysis of retinal phenotypes in single and double mutants in addition to heterologous yeast studies to determine *bgm* and *dbb* biochemical function revealed an overlapping but not identical function of these two highly related genes. In Chapter 3, we then use the individual single mutant animals to elucidate the biological roles of the ACSL pathway in the nervous tissue, revealing important lessons that provide insight into the mechanism of ALD pathology.

Therapeutic approaches for ALD

Therapies for ALD have been lacking. A dietary treatment (popularized in the movie Lorenzo's Oil) was developed to eliminate accumulated VLCFA levels in affected individuals (Rizzo et al., 1989). However, this therapy was discontinued after multiple reports of its inefficacy and harmful effect on platelet levels in some patients (Moser, 1999; van Geel et al., 1999; Zierz et al., 1993; Zinkham et al., 1993). Currently, the only potentially effective therapy (bone marrow transplant) is applied to only the most severe cases, as this treatment is itself associated with a 20% mortality rate and degeneration is, at best, halted from progressing and not reversed (Aubourg et al., 1990; Shapiro et al., 2000). Experimental therapies using patient hematopoietic stem cells virally transduced with a functional copy of *ABCD1* have been reported with some success. However, this therapeutic approach has yet to be approved by the Food and Drug Administration in the United States (Cartier et al., 2009). In the end, there are no currently feasible therapeutic options for ALD. In Chapter 3, I revive the use of dietary therapy to understand the cellular defect leading to neurodegeneration in *bgm* and *dbb* animals. This led to dietary

reduction in the age-progressive neurodegenerative phenotype, showing promise for future dietary therapeutic approaches in humans.

Lipid Products

Activation of lipids and shunting to specific functions

The enzymatic reaction that ACSs control represents a crucial step of cellular lipid metabolism and act as gatekeepers for lipid bioavailability. Lipid acylation by ACSs occurs in both the *de novo* lipid synthesis pathway and the reacylation pathway, which recycles lipids from other cellular uses. Through this activation step, acyl CoA synthetases have historically been viewed as masters of lipid partitioning due to their ability to shunt lipids to a specific function within a cell (Coleman et al., 2002). Pharmacological studies using incomplete inhibitors of the ACS family have revealed these independent and specific roles of ACSs within a cell. These observations were first made using the drug Triacsin C—a fungal-derived competitive ACS inhibitor (Tomoda et al., 1987). The following studies take advantage of differences between *de novo* synthesis versus reacylation of lipids for the generation of triacylglycerol and phospholipid product. *De novo* synthesis of these two lipid products incorporates glycerol in the form of glycerol-3-phosphate that serves as a backbone for which fatty acyl-CoAs can be attached. Using recycled fatty acids for these lipid products via the reacylation pathway, however, does not incorporate glycerol, as lipid substrates in this pathway have already been modified. When Triacsin C was applied to human skin fibroblasts, radiolabeled glycerol incorporation into phospholipid and triacylglycerol products was significantly blocked signifying disruption of the *de novo* pathway.

However, when radiolabeled oleate was used to measure input of both the *de novo* pathway and the reacylation pathway into these lipid products, only the incorporation of oleate into triacylglycerol was blocked. That oleate incorporation into phospholipids was intact suggests ACSs in the *de novo* pathway are separate from the reacylation pathway and that the latter ACS pool is triacsin C-insensitive and accounts for the majority of phospholipid product (Igal et al., 1997). Further observations of selective ACS inhibition have been confirmed and extended using *in vitro* methods (isolated rat hepatocytes and generation of recombinant protein) with triacsin C and a different drug, troglitazone (Fulgencio et al., 1996; Kim et al., 2001). Of note, the mechanism of action of troglitazone on ACSs is through activation of PPARγ and not through competitive inhibition as it is with triacsin C, thus, not all ACSs lie downstream of PPAR activation (Lambe and Tugwood, 1996). This idea of separate ACS pools is consistent with known subcellular localization patterns of different ACSs within the ER, mitochondrial, and microsomal membranes or floating freely in cytosolic pools (Coleman et al., 2000; Krisans et al., 1980; Lewin et al., 2001). Although this observation is consistent with ACS pools with specific function, it is difficult to parse drug specificity from the possibility of drug partitioning in specific locations in the cell and therefore only acting on specific ACSs. Additionally, ACSs have been found to display sometimes-overlapping functions that complicate functional studies due to redundancy. In Chapter 2, we characterize the first genetic analysis of an ACS double mutant and through comparison of retinal phenotypes in single and double mutants, identify both independent and overlapping functions of the Drosophila ACSs *bgm* and *dbb*.

Still, the ability of ACSs to shunt lipids into specific cellular functions has also

been investigated using gain- and loss-of-function studies can provide insight that cannot be explained by redundancy. Overexpression of rat ACSL1 in primary hepatocytes channeled radiolabeled oleate into diacylglycerol and phospholipids, decreased incorporation into cholesterol esters and secreted triacylglycerol, and did not affect oleate incorporation into beta-oxidation, stored triacylglycerol, or total metabolized oleate (Li et al., 2006). In adipose-specific disruption of ACSL1 in mice, a specific role of ACSL1 for shunting fatty acids into mitochondrial beta-oxidation is seen, although this specific result could be explained by redundant compensation of all other functions except shunting to beta-oxidation (Ellis et al., 2010). Nonetheless, it displays a requirement for ACSL1 in shunting fatty acids into energy utilization pathways in adipocytes: whether this is the only function of ACSL1 remains to be determined. In studies using human hepatoma cells, RNA interference targeting ACSL3 did not significantly decrease total ACS activity, but significantly affected radiolabelled oleate incorporation into phosphatidylcholine suggesting activation by ACSL3 shunts lipids into phosphatidylcholine synthesis (Yao and Ye, 2008). Therefore, ACSs may still play a role in partitioning fatty acids into specific functions within a cell. However, parsing a "shunting" role from redundant function in the ACS family of proteins must be considered for each case.

The Fatp example of lipids required for function

One landmark finding with particular relevance to the studies described in this dissertation is the discovery of a role of *Drosophila fatp* in neuromaintenance in the adult eye (Dourlen et al., 2012). *fatp*—a membrane protein with dual functions as an acyl-CoA

synthetase and fatty acid transporter—was identified in a P-element screen to uncover genes required for photoreceptor differentiation, survival, and polarity—*fatp* is specifically required for neuromaintenance (Gambis et al., 2011). Mosaic retinas with $fatp^{k10307}$ clones experience age-dependent photoreceptor loss beginning around day 4 post-eclosion and peaking around day 15 post-eclosion. Degeneration in these animals is cell-autonomous and rescued by neuronal expression of a dominant-negative *rh1* allele ($rh1^{G69D}$) and light dependent. These data pointed to a neurodegenerative mechanism that is dependent on the phototransduction response in photoreceptors. In Chapters 2 and 3, we describe a strikingly similar neurodegenerative phenotype (with respect to age of onset, ultrastructural phenotype, and light dependency) in our ACS mutants. The loss-of-function phenotype shared by null alleles of *fatp* and the two ACS genes *bgm* and *dbb* suggest that the *fatp* mechanism of neurodegeneration is due to its function as an ACS and not as a fatty acid transporter. In Chapter 4, I investigate whether rhodopsin recycling plays an integral role in the neurodegenerative phenotypes associated with loss of *bgm* and or *dbb*.

Sources and uses of cellular VLCFAs

ALD is thought to result from defects in processing very long chain fatty acids, as evidenced by the accumulation in plasma and affected tissues (Igarashi et al., 1976). We show in Chapter 2 that the long/very long chain family of lipids serves as substrates for the *Drosophila* Bgm and Dbb proteins. Therefore, this dissertation will maintain a particular focus on the roles of the long/very long chain fatty acids.

Fatty acids vary in chain length as well as saturation leading to a diverse

repertoire of lipids. Consistent with tissue-specific roles for specific FAs within the cell, tissue compositions of FAs vary according to the tissue type (Carvalho et al., 2012; Sassa and Kihara, 2014). As previously described, cells obtain fatty acids through one of two pathways (or a combination of both): 1) import and activation by cellular ACSs, or 2) modification of endogenous fatty acids by elongases (lengthen acyl chain) or desaturases (shorten acyl chain) (see Figure 1.3 B). With regard to very long chain fatty acids (>C22), the relative contribution of each of these pathways to cellular concentrations is unknown and may additionally be tissue-specific. Evidence exists that utilization of dietary fatty acids could account for a majority of VLCFAs in the brain. However, the methods do not discriminate between the two sources above. The only study focusing on the source of VLCFAs was performed on a terminally ill human subject by feeding radiolabelled VLCFAs and measuring incorporation of label in brain tissue upon autopsy (Kishimoto et al., 1980). Ninety-six percent of the C26:0 fatty acids in the sampled brain tissue contained radiolabel. This suggests dietary VLCFAs contribute to at least C26 composition in vivo; however, due to the duration of time the patient was fed radiolabel (100 days), and the fact that radiolabel incorporation was only measured for C26 at a single timepoint, it is difficult to determine if these fatty acids were activated and used as C26, or if the radiolabelled fatty acids undergo multiple conversions to other fatty acid species and, in fact, account for 96% of all fatty acids (or other specific species) in the brain. Dietary modulation of *in vivo* VLCFAs composition has also been documented in *Drosophila* (Carvalho et al., 2012).

Despite knowledge of the existence of the very long chain class of fatty acids since the late 1980's, VLCFAs have been fairly understudied. Under recognition of

VLCFAs could be due to their low abundance in tissue isolates (~2% of retinal lipids) (Agbaga et al., 2010; Aveldano, 1987; Aveldano and Sprecher, 1987; Rotstein and Aveldano, 1988). The highest concentrations of VLCFAs are found in the testis, brain and retina (Agbaga et al., 2010). Despite low abundance, multiple lines of evidence point to the biological relevance of this class of fatty acids in at least some tissues. This evidence is gleaned from mutant phenotypes in elongase genes that generate VLCFAs, suggesting that contribution from at least the second pathway above contributes significantly to total VLCFA levels. In mice lacking *Elovl3* and *Elovl1*, skin barrier function is significantly compromised as a proposed consequence of altered L/VLCFA synthesis (*Elovl3* -/- mice displayed decreased C16 and C18 and increased C20 whereas *Elovl1* -/- mice displayed increased <C24 and decreased >C24) (Sassa et al., 2013; Westerberg et al., 2004). *Elovl3* mutant mice were susceptible to hypothermia after submersion in water. *Elovl1* mutant mice were lethal within 24 hours of birth. *Drosophila* mutants in the elongase gene *bond* show spermatogenesis defects due to an inability for furrow ingression during cytokinesis. This is proposed to be due to an inflexibility of the plasma membrane to bend as the furrow progresses in the final stages of cell division (Szafer-Glusman et al., 2008). Spermatogenesis defects are also seen in mice lacking *Elovl2*. Both homozygous mutant as well as heterozygous animals have decreased VLCFAs C28 and C30 in the testis (Zadravec et al., 2011). In humans, mutations in the *ELOVL4* gene cause a rare form of macular dystrophy (Stargardt macular dystrophy 3)(Agbaga et al., 2010; Zhang et al., 2001). Of note, inherited alleles are haploinsufficient as individuals heterozygous for *ELOVL4* manifest disease that could either suggest a sensitivity of retinal cells to VLCFA levels or could be explained through

a feedback mechanism that down regulates expression. Fitting with a requirement of VLCFAs in neuromaintenance, expression of ELOVL4 is high in photoreceptors of the mouse retina and increases with age until postnatal day 30 where it remains at a steady state level (Mandal et al., 2004). These mutant phenotypes suggest a requirement in multiple tissues for VLCFAs, including the neuronal tissue of the retina.

Defects in lipid metabolism are linked to neurodegenerative disease

ALD stems from alterations in lipid homeostasis, as genetic variants in the lipid transporter ABCD1 are disease-causing in families with X-linked ALD, the only form of ALD with a tractable genetic cause. Other nonleukodystrophy neurodegenerative diseases are caused by variants in genes encoding proteins that like ABCD1 play key roles in lipid metabolism (acid sphingomyelinase: Niemen Pick type A OMIM#257200, alpha-galatosidase A deficiency: Fabry disease OMIM#301500, and acid ceramidase: Farber's disease OMIM#228000). Although all of these diseases are associated with an accumulation of lipid species, the mechanism of neurodegeneration is unknown (Ahmad et al., 2009; Madra and Sturley, 2010; Ries and Gal, 2006; Schiffmann, 2006; Vazquez et al., 2012; Zhang et al., 2000). Fabry disease and Farber's disease (like ALD) both display variable expressivity suggesting as-yet-unidentified genetic or environmental modifiers. That these diseases, along with ALD and undiagnosable leukodystrophies, remain so poorly understood demonstrates the need for increasing our basic understanding for the roles lipids play in maintenance of the nervous system.

Cell Specificity in Neurodegenerative Diseases

Cell autonomous and noncell autonomous mechanisms

Determination of cell types affected in ALD opens the door to understanding diseases etiology and treatment. Whether the cellular defect is autonomous in neuronal cells, or nonautonomous in glial cells, contributes a great deal to understanding the requirements of these cell types for neuromaintenance and by deduction, the mechanism of dysfunction in neurodegeneration. Additionally, therapeutic protocols are depedent upon cell type(s) affected, as mechanisms for therapeutic delivery are unique for each (Drinkut et al., 2012). Both the glial and neuronal cell types that constitute the nervous system have been implicated in various neurodegenerative diseases. Glia are fatty-rich support cells that have recently been recognized as playing an important role in Alexander's Disease (Wang et al., 2011a), possessing the ability to perform immune-like function and required for general neuromaintenance (Liu et al., 2015; Mrak and Griffin, 2005). However, it is the metabolically demanding neuronal cell types that have historically been studied as the primary cells affected in neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Forloni et al., 1993; Mironov et al., 2003; Nikolaev et al., 2009; Obeso et al., 2010). Overall, the relative contribution of each of these cell types is difficult to resolve in human patients (Barker and Cicchetti, 2014). In ALD, there is little known concerning the primary cell type affected. Some reports suggest that glial cells (most often oligodendrocytes and astrocytes) are dysfunctional in ALD patients or *ex vivo* upon increased VLCFA treatment (Aubourg and Dubois-Dalcq, 2000; Di Biase et al., 2004; Feigenbaum et al., 2000; Katsuragi et al., 1996; Khan et al., 1998) whereas others focus on the function of ABCD1 or ACSs in neurons (Pei et al.,

2003). Still, some studies suggest both neuronal cells and glia are dysfunctional (Hein et al., 2008). The involvement of non-nervous system tissue also adds complexity to an already complicated disease, begging the need for a clean model in which to test tissue-specific requirements of ACSL metabolism early in disease-progression. The ability to test whether defects in either neurons or glia cause neurodegeneration in an *in vivo* ALD model has not been possible until recently (Sivachenko et al., 2015).

Research Objectives

Despite a growing understanding of ALD pathology and the identification of *ABCD1* as a causative gene, the mechanistic role of ABCD1 in neuromaintenance remains to be definitively shown. Further, effective disease-modifying therapies are not yet available. The work presented in this dissertation describes two approaches to address these challenges. The first objective is to define the timing and cell type critical for ALD neuropathology. In Chapter 3, I show that *bgm* deficient flies expressing a rescue construct solely in adult neuronal cells rescues neurodegeneration. Additionally, I show that *dABCD1* knock down in neuronal cells but not glial cells results in a neurodegenerative phenotype. The second objective is to determine the subcellular mechanism of neurodegeneration in *bgm* and *dbb* mutant animals. In Chapter 3, through the use of two dietary regimes, I differentiate between the ideas that a toxic accumulation of precursor or a lack of terminally processed lipid product leads to neurodegeneration. I show that *bgm* and *dbb* mutant flies fed a diet high in fatty acids presumed to accumulate show no worsening of neurodegeneration, while those flies fed a diet containing fatty acids that can be converted to the terminally processed product show a significant rescue

of neurodegeneration. I confirm these observations by artificially decreasing the cellular need for this terminally processed product by decreasing light exposure in adult *bgm* and *dbb* mutants and show a rescue of neurodegeneration in animals raised in constant darkness. Finally, I test the requirement for this terminally processed lipid product by down regulating anther biochemical pathway used to obtain this product—the elongase pathway. I show that animals harboring RNAi targeted to the elongase encoded by *CG2781* share a neurodegenerative phenotype seen in other members of the ACSL pathway. I interpret these results to suggest a revival of dietary therapy, and combined with further resolution of the specific cellular defect expect that a long-term outcome of my studies will be identification of potential avenues for therapy to the incurable disease ALD.

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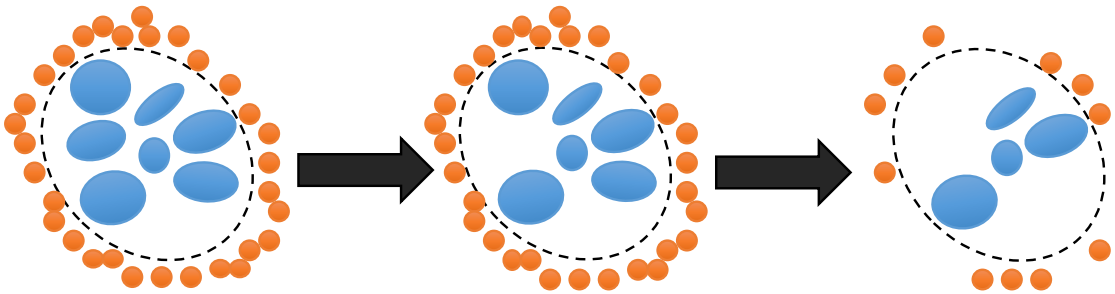
Figure 1.1 Scope of research objectives. The goals of this dissertation are to contribute to two fundamental questions centered around the use of fatty acids in the nervous system which represent untested questions in the field of ALD research. (A) The first is whether the primary cell type affected is a neuronal or glial cell, as these represent very different cellular populations with respect to etiology as well as therapies. (B) The second is identifying whether a buildup of precursor or a lack of activated fatty acid product results in the subcellular defect leading to dysfunction.

Scope of research objectives

A. Primary cell type affected: *bgm* is required in neurons of the retina



B. Subcellular defect: a lack of activated fatty acid product and not an accumulation of precursor underlies cellular defects in ACS mutants

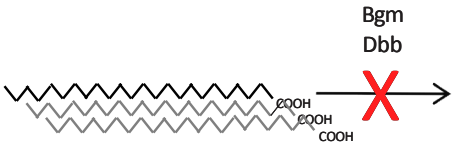
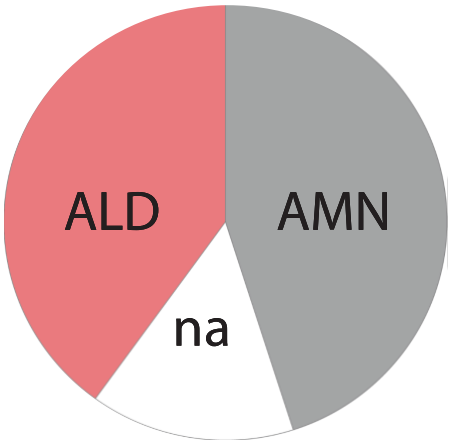


Figure 1.2 Increased VLCFAs do not account for phenotypic presentation of ALD. (A) Spectrum of neurological phenotypes in patients with the most common cause of ALD—*ABCD1* mutations—paired with (B) the percentage of patients displaying a significant increase in plasma VLCFA levels. Merging these pie charts highlights patients who harbor an increase in VLCFAs without displaying any neurological phenotype.

A



B

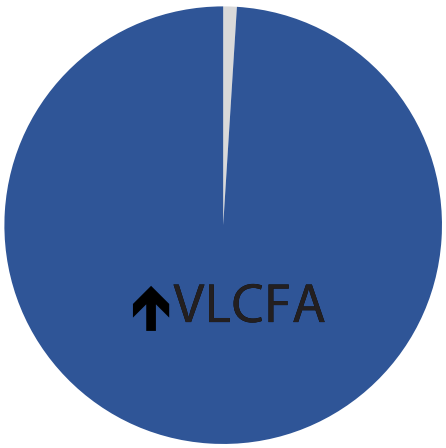
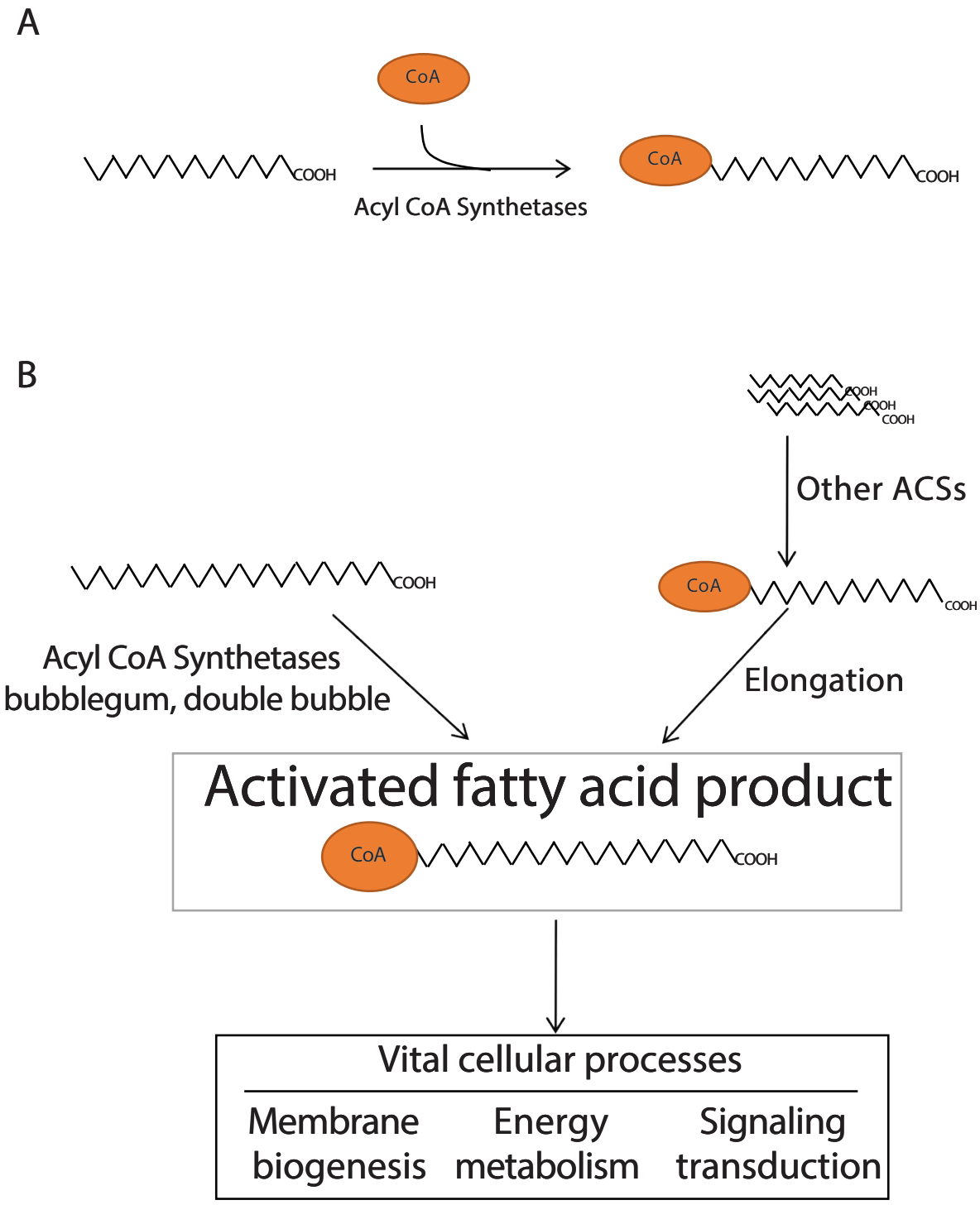


Figure 1.3 Obtaining activated fatty acids. (A) The family of Acyl CoA Synthetases biochemically activate fatty acids by esterifying the acyl chain with a coenzyme A. Only activated fatty acids are biochemically available for further modification and use within the cell. (B) Activated VLCFA product is obtained through two methods—activation of free VLCFAs by Acyl CoA Synthetases specific for long and very long chain fatty acids or elongation of fatty acids with shorter carbon chains through a four-step elongation pathway.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Acyl CoA Synthetases
bubblegum, double bubble
Elongation
Activated fatty acid product
CoA
COOH
Vital cellular processes
Membrane biogenesis
Energy metabolism
Signaling transduction

CHAPTER 2

NEURODEGENERATION IN A *DROSOPHILA* MODEL OF ADRENOLEUKODYSTROPHY: THE ROLES OF THE BUBBLEGUM AND DOUBLE BUBBLE ACYL-COA SYNTHETASES

Abstract

Debilitating neurodegenerative conditions with metabolic origins affect millions of individuals worldwide. Still, for most of these neurometabolic disorders there are neither cures nor disease-modifying therapies, and novel animal models are needed for elucidation of disease pathology and identification of potential therapeutic agents. To date, metabolic neurodegenerative disease has been modeled in animals with only limited success, in part because existing models constitute analyses of single mutants and have thus overlooked potential redundancy within metabolic gene pathways associated with disease. Here we present the first analysis of a very long chain acyl-CoA synthetase double mutant. We show that the *Drosophila bubblegum (bgm)* and *double bubble (dbb)* genes have overlapping functions, and that the consequences of *bubblegum double bubble* double knockout in the fly brain are profound, affecting behavior and brain morphology, and providing the best paradigm to date for an animal model of

Adrenoleukodystrophy (ALD), a fatal childhood neurodegenerative disease associated with the accumulation of very long chain fatty acids. Using this more fully penetrant model of disease to interrogate brain morphology at the level of electron microscopy, we show that dysregulation of fatty acid metabolism in vivo is causal of neurodegenerative pathologies evident in both neuronal cells and their support cell populations, and leads ultimately to lytic cell death in affected areas of the brain. Finally, in an extension of our model system to the study of human disease, we describe our identification of a leukodystrophy patient who harbors a rare mutation in *SLC27a6*, a human homologue of *bgm*.

Introduction

Adrenoleukodystrophy

Adrenoleukodystrophy (ALD) is a rare and oftentimes fatal progressive neurodegenerative disease. The most common form of the disease is X-linked (X-ALD) with an estimated incidence of 1:17,000 in all ethnic groups. X-ALD is a clinically heterogeneous disorder, exhibiting incomplete penetrance and variable expressivity (Moser et al., 2005a).

The most severe form of X-ALD, affecting ~40% of all patients, is cerebral ALD. Demyelination in the central nervous system (CNS) constitutes the major burden of cerebral ALD, with the disorder most frequently diagnosed as progressive neurological dysfunction in previously healthy boys between the ages of 4 and 8 years old. A less severe, but nonetheless debilitating, form of the disease (Adrenomyeloneuropathy [AMN]) occurs in ~40% of X-ALD patients. In AMN patients, demyelination is

confined (at least initially) to the peripheral nervous system (PNS). AMN patients present in the second to fourth decade of life with impotence, sphincter dysfunction, and slowly progressive paraparhesis. About 10% of X-ALD patients have adrenal insufficiency without nervous system involvement; a similar small number of males remain entirely asymptomatic. It is notable that wide phenotypic variability is observed within kindreds segregating the same mutation; thus, environmental and/or genetic factors must play significant roles as disease modifiers (Moser, 1997).

Function of the ATP-binding-cassette transporters in ALD

The gene responsible for X-ALD has been cloned and shown to encode a peroxisomal half ATP-binding-cassette transporter (*ABCD1*: ATP-binding cassette, sub-family D [ALD], member 1) (Mosser et al., 1993). The 745 amino acid ABCD1 protein (or ALDP: Adrenoleukodystrophy Protein) localizes to the peroxisomal membrane, where like other members of the ABC (ATP-binding cassette) transporter family, it functions to transport very long chain fatty acids (VLCFAs, 22 or more carbons) via their acyl-CoA esters into peroxisomes where they are degraded by ß-oxidation (Fig. 1A). Indeed, accumulation of saturated VLCFAs is a known biochemical hallmark of X-ALD (Igarashi et al., 1976). All X-ALD patients, including asymptomatic carriers, show elevated levels of VLCFAs, in particular tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0) in the plasma, brain, and adrenal gland. There is more recent evidence for the additional accumulation of monounsaturated VLCFAs in plasma and fibroblasts of X-ALD patients (Kemp et al., 2005). This finding has especially important consequences with respect to treatment of individuals with the highly controversial Lorenzo's oil, itself

a mixture of mono–unsaturated VLCFAs.

Function of very long chain acyl-CoA synthetases in ALD

Acyl-CoA synthetases (ACSs), which function immediately upstream of ABC transporters in effecting peroxisomal degradation of fatty acids, have also been implicated in ALD disease pathology (see Fig. 2.1A). Chemically-reactive fatty acyl-CoA esters, which are derived from their chemically-inactive precursors (free fatty acids) in a two-step process, serve as substrate for the ABCD1 transporter. It is the second step of the fatty acid esterification process that is catalyzed by ACS. There are four recognized enzyme families with ACS activity, those activating short (2-5 carbons), medium (6-12 carbons), long (13-21 carbons), and very long (≥22 carbons) fatty acid substrates. Although families are defined by substrate specificities, it is common for these to overlap between families, particularly with respect to the long- and very long-chain substrates (Lu et al., 2009; Steinberg et al., 1999; Watkins, 2008; Watkins et al., 2007). The essential role of ACSs in fatty acid metabolism is supported by their high degree of conservation in organisms ranging from bacteria to humans. ACSs are defined by two highly conserved functional domains: the 10 amino acid AMP–binding domain and the 35 amino acid signature motif that plays an integral role in determining substrate specificity (Watkins, 2008).

Animal models of ALD

Investigators have long sought to resolve the clinical heterogeneity of ALD in animal models, thus far however with only very limited success. Phenotypes associated

with knockout of *ABCD1* in mouse are dependent upon genetic background. In one *ABCD1* knockout mouse model of X-ALD, mice experience a normal life span and exhibit no signs of degeneration in either the CNS or PNS. The same knockout in a different genetic background, however, does lead (albeit at low frequency) to accumulation of VLCFAs and development of AMN-type symptoms (Forss-Petter et al., 1997; Lu et al., 1997). With regard to ACS loss-of-function, neither neurological defects nor VLCFA accumulation is detected in VLCS-1 (VLCFA acyl-CoA synthetase) knockout mice (Heinzer et al., 2003).

Data obtained in a fly model of ACS function are more encouraging, as mutation of *bubblegum* (*bgm*), the gene encoding a *Drosophila* long chain ACS (ACSL) with predicted long- and very long-chain substrate specificities results in an autosomal recessive neurodegenerative phenotype (Min and Benzer, 1999b). *bgm* homozygotes exhibit impaired vision and a "bubbly" optic lobe, phenotypes resulting from a loss of nervous tissue. The documented *bgm*-associated biochemical and neurodegenerative abnormalities are subtle, being limited to moderate accumulation of two saturated VLCFAs and minimal disorganization and degeneration of the optic lobe. *bgm* flies have, therefore, not been embraced as a strong animal model for ALD.

Until now, what none of the ABC transporter or ACS animal studies (neither fly nor mouse) have adequately contemplated is the considerable potential for redundancy within these gene families. With regard to the latter, although original classifications of the acyl-CoA synthetases were based solely on the fatty acid chain lengths of their substrates, more recent studies point to overlapping substrate specificities (Watkins, 2008; Watkins et al., 2007). Indeed, having confirmed that the *bgm* allele used by Min

and Benzer (1999b) is a null, we show here that a far more profound phenotype, and thus a better ALD disease model, is found in double mutant animals harboring mutations not only in the *bgm*-encoded ACSL, but also in its homologue the *double bubble* (*dbb*)-encoded ACSL.

As described in this report, our focus on the similarities between *bgm dbb* and ALD phenotypes provides new insights into defects associated with accumulation of VLCFAs. In particular, we show that defects in VLCFA metabolism affect neurons as well as their support cells, and lead to widespread cell losses in the fly brain. In addition, our detection of inclusions in *bgm dbb* mutant flies augurs an attractive diagnostic tool. Underscoring the relevance of ACSL mutations to ALD phenotypes, we also describe our identification of a young boy with an undiagnosed leukodystrophy harboring a mutation in SLC27a6, a dual ACSL/fatty acid transporter with homology to Bgm and Dbb.

Materials and Methods

Fly stocks

Balancers, Canton S, *w1118* and *Df(bgm)* (*Df(2L)b87e25/CyO* [34B12-C1;35B10-C1]) have been described (FlyBase, 2003). *bgm1* was from K-T. Min (NINDS). Strains for targeted mutagenesis were from K. Golic (Univ. of Utah).

Targeted mutagenesis

Ends-out gene targeting was performed as described (Gong and Golic, 2003). Our targeting construct was engineered to delete the 5' two-thirds of the *dbb* coding region. To this end, DNA fragments corresponding to 14023363-14026942 and

14028337-14031358 in the *Drosophila melanogaster* genome were subcloned into the pW25 plasmid. Targeting was verified in progeny by PCR (using 5'-CGAAAGGGAGAGTTCGACTCC and 5'-GGC TGCTGCAATAAGATTAGGGC primers) and by RT-PCR (using 5'-CGGATATCACACTGTCAC and 5'-CATCGGCATACATCTTGGACA for *bgm,* 5'-GCCATCATCCTTTTCACAGAC and 5'-GTACAGACGCTCGATGACTTTG for *dbb*, and 5' GTGCCGAATACATCGTGGAG and 5'- AACGACCTCCTCATCGGTGT for *Gapdh2*).

Yeast rescue

BY4743 strains mutant for the yeast genes FAA1 and FAT1 (the generous gift of Diane Ward Univ. of Utah) were transfected with constructs containing *Drosophila bgm* or *dbb* cDNAs cloned into the yeast expression vector p426-ADH1. Yeast cultures were grown on YPD (yeast peptone dextrose; standard growth medium) supplemented with cerulenin (Cayman Chemical, Ann Arbor, MI) to inhibit *de novo* synthesis. Oleate and myristate were obtained from Nu Check Prep (Elysian, MN). G418 selection was employed to monitor the FAA1 and FAT1 mutant backgrounds and 5-FOA to monitor the p426-ADH1 vector.

Activity and lifespan studies

Wild-type and mutant flies were maintained in a 12h:12h light:dark cycle at 25°C. For activity phenotyping, 6-18 males/genotype were individually transferred to monitor tubes on the day of eclosion, and to fresh tubes weekly. Locomotor activity was

monitored using the *Drosophila* Activity Monitoring System starting within 24 hours of eclosion and continuing each week until day 30 post-eclosion (Trikinetics, Waltham, MA). Average peak activity was determined by analyzing beam break occurrences during the 2 hours before and after light change after acclimatization into the activity chambers. For longevity studies, 40-100 flies/genotype were seeded at an initial density of 20 flies/vial. Survivors were transferred to fresh vials every 7 days. Curves are estimated from the results of three independent trials. Graphpad Prism software was employed for statistical analyses.

Histology and transmission electron microscopy

For histological examination, heads were dissected and processed as described (Palladino et al., 2002). Serial 6-µm sections were stained with hematoxylin and eosin, and scored blindly under a light microscope for the presence of vacuoles with diameter greater than 3 µm, retinal degeneration, and fenestrated membrane changes. For TEM studies, heads were embedded in Spurr's epoxy resin, sectioned at 70-90 nm thickness using a Reichert-Jung Ultracut E microtome, postfixed with osmium and stained with 2% uranyl acetate and Reynolds' lead citrate. Ultrastructural analysis was carried out using a Hitachi H-7100 electron microscope at 125 kV.

Fatty acid profiling

Extracts from 18-day-old males and females were used for purification and methylation of fatty acids. Solutions were analyzed by GC-MS using a Micromass GCT Premier time-of-flight mass spectrometer fitted with an Agilent 6890 gas chromatograph

and autosampler (Waters Corp., Beverly, MA).

Patient analysis

The Institutional Review Boards of the University of Utah and Intermountain Healthcare (IH) approved this work. Clinical exome sequencing was performed through Baylor Medical Genetics Laboratories. Confirmatory Sanger sequencing for *SLC27a6* was performed using primers (5'-3'): TGAGCAAACATGTTCTAGACAGAG and reverse-TACCTTTGTTACTGGCGGGT. Template DNA was isolated from cheek cells in all individuals shown in the pedigree.

Results

bgm and *dbb* encode duplicated *Drosophila* acyl-CoA synthetases

Our studies have their foundation in the postulate that acyl-CoA synthetases in *Drosophila* have overlapping and hence sometimes redundant function. In this regard, there are multiple ACSs in all species. In *Drosophila*, in addition to *bgm,* there are seven genes encoding fatty acid acyl-CoA synthetases (Fig. 2.1B). *bgm* and CG4500 (designated here as *double bubble* [*dbb*]) are more closely related to one another than they are to the other members of their family. *bgm* and *dbb* share 43% identity and 62% similarity at the amino acid level. Furthermore, *bgm* and *dbb* map immediately adjacent to one another in the *Drosophila* genome (7.7 kb apart; Fig. 2.1C). The *bgm* and *dbb* genes belong to the evolutionarily well-conserved Bgm family of ACSLs. The human homologue (hBgm) is 41% identical (61% similar) to *Drosophila* Bgm, and 37%

identical (55% similar) to *Drosophila* Dbb (supplementary material Fig. 2.S1).

The *bgm* and *dbb* gene products retain their predicted acyl-CoA synthetase activity

To test whether the *bgm* and *dbb* gene products retain their predicted acyl-CoA synthetase activity, as well as to determine whether they possess the additional fatty acid transport function that defines the related FATP family of dual functioning enzymes, we assessed *bgm* and *dbb* activity in a yeast heterologous system. Expression of either the *bgm* or *dbb Drosophila* cDNA in an *S. cerevisiae* strain that is null for Faa1p (the major ACSL in yeast) restored strain viability in the presence of the *de novo* fatty acid synthesis inhibitor cerulenin when myristate was added to the culture medium; only *bgm*, however, rescued in the presence of oleate (Fig. 2.1D). (Both myristate and oleate represent long-chain fatty acids, C14:0 and C18:1, respectively). In contrast, neither *Drosophila* cDNA was able to rescue Fat1p (fatty acid transporter) -dependent lethality in the presence of cerulenin (data not shown). Thus, while both *bgm* and *dbb* retain acyl-CoA synthetase activity and exhibit overlapping substrate specificities, neither is dual functioning. The latter finding was somewhat unexpected as mouse ACSL1 (mouse Bgm) restores not only fatty acid activation to Faa1p-deficient yeast but also fatty acid uptake to Fat1p mutants, indicating a concordance of activation and uptake functions in this enzyme (DiRusso et al., 2008).

Knocking out *bgm* and *dbb* gene functions

Extensive Bgm/Dbb homology at the protein level, as well as their overlapping substrate specificities led us to speculate that the *bgm* and *dbb*-encoded ACSL functions are redundant. We tested this hypothesis in genetic analyses of single and double mutants. We used the previously characterized bgm^{1} null allele for our study, but turned to targeted knockout methods to generate the dbb^{1} null allele and the bgm^{1} dbb^{1} double null mutant (Fig. 2.2A). PCR analysis allowed discrimination of wild-type and mutated loci based on a 3.3 kb fragment differential at the dbb^{1} locus (Fig. 2.2B). Measurement of transcript levels in wild-type and mutant animals revealed traces of *bgm* mRNA in the previously-characterized bgm^{1} null background, but no detectable *dbb* mRNA in the genetically-engineered dbb^{1} null (Fig. 2.2C).

bgm and *dbb* function redundantly in the adult CNS

To study the functional relationship between *bgm* and its homologue *dbb* in maintenance of adult CNS integrity, we analyzed brain morphologies in wild-type flies, and in single and double mutant flies. For histological studies, heads from 18-day-old wild-type (Canton S) and mutant (bgm^{1}, dbb^{1} and bgm^{1} dbb^{1}) flies were embedded in paraffin, stained with hematoxylin and eosin, and scored blindly under a light microscope for the presence of: (a) optic ganglion (laminal) holes with diameters greater than 3 μm, (b) retinal disorganization, and (c) fenestrated membrane loss. While the optic ganglion phenotype was quantified by hole number, the degree of retinal degeneration was scored qualitatively as 1 for normal appearance, 2 for mild tissue loss, 3 for moderate degeneration, and 4 for severe degeneration. For the assessment of fenestrated membrane

morphologies, we scored images from 1 (normal) to 5 (very severely damaged) taking into account membrane thickness, presence of gaps, displacement of photoreceptor cells into the optic lobe, as well as regularity of the basement membrane. To obtain the mean score for each animal, we analyzed at least eight hemibrain sections from the same fly. Data from five flies per genotype were analyzed by ANOVA and Welch Two Sample *t*-tests.

Although fully viable, dbb^1 and bgm^1 dbb^1 adults exhibit clear central nervous system defects, suggesting that *dbb* (like *bgm*) is required for maintenance of adult nervous system tissue (Fig. 2.3A-F). We documented laminal holes in bgm^1, $bgm^1/Df(bgm)$, dbb^1 and bgm^1 dbb^1 18-day-old mutant flies but not in age-matched wild-type controls (Fig. 3I), nor in newly eclosed mutants as was previously shown by Benzer. We observed no significant differences in the degree of degeneration of the lamina of age-matched bgm^1 homozygotes and $bgm^1/Df(bgm)$ *trans* heterozygotes ($p = 0.13$), thereby genetically establishing the amorphic character of the bgm^1 allele. Statistical analyses revealed differences in hole numbers between single mutants and wild types to be highly significant ($p \leq 0.001$); thus each gene is individually required for neuronal maintenance. Holes were most abundant in bgm^1 dbb^1 double mutants ($p < 0.0002$ for comparisons both to wild types and single mutants). Thus, these data indicate: (1) that in the laminal region of the brain, the *bgm* and *dbb* genes function redundantly, and (2) that the double mutant animal is fully penetrant and provides a more powerful resource (than either of the single mutants alone) for determining the effects of fatty acid dysregulation in neurodegeneration.

Histological examinations of hematoxylin-eosin stained brain sections revealed

that in addition to laminal degeneration, which had been documented although not previously quantified for *bgm* (Min and Benzer, 1999b), both single and double mutants also exhibit phenotypes not previously described. In this regard, we observed retinal degeneration, as well as thinning and irregularity of the fenestrated membrane—the structure defining the anatomic border between the retina and lamina—in bgm^1, dbb^1, and bgm^1 dbb^1 mutant animals. That the aberrantly-constructed fenestrated membrane in *ACSL* mutants is functionally encumbered is most evident in double mutant animals where retinal nuclei can routinely be seen crossing from the retina into the lamina (Figure 2.3G,H). In quantifying retinal and fenestrated membrane defects (Fig. 2.3J,K), we found that degenerative changes are stronger in the bgm^1 mutant than in the dbb^1 mutant ($p \leq 0.02$), although in comparisons to wild type, it was clear that *dbb* does contribute in significant fashion to retinal and fenestrated membrane structure ($p = 0.015$ and $p = 0.00017$, respectively). Our comparisons of retinal and fenestrated membrane degeneration in bgm^1 and bgm^1 dbb^1 mutant animals did not reveal statistically significant differences between the two ($p = 0.34$ and $p = 0.54$), but as is true for ALD in humans, wide phenotypic variability was observed in double mutants, and the highest levels of degeneration were documented in double mutants only. Taken together comparisons of wild-type and mutant retinal and laminal brain sections demonstrated that: (1) loss of the *bgm* and/or *dbb*-encoded ACSL results in statistically significant levels of nervous system impairment in all regions tested, and (2) cooperativity between the two genes varies by brain region - indicative of both overlapping (laminal) and cell-selective (retinal) outcomes for this gene pair.

Mutations in *bgm* and *dbb* affect lipid- and membrane-rich pigment cells

To gather insight into the cellular origin of the *bgm* and *dbb* neurodegenerative phenotypes, we next turned to transmission electron microscopy (TEM). We focused first on the fenestrated membrane, which forms the physical and electrical barrier between the eye and the brain. As suggested by our initial hematoxylin-eosin-based analysis, we observed a substantial decrease in the extracellular component of the fenestrated membrane in *ASCL* mutants (Fig. 2.4A-D). Ultrastructural imaging of wild-type and mutant eyes from 18-day-old adults further revealed retinal holes, disarray in the normal hexagonal pattern of ommatidial structure, and perhaps most tellingly, loss of pigment cells (the non-neuronal support cells that surround and insulate single retinal ommatidial clusters) in mutants (Fig. 2.4E,F).

The most parsimonious explanation for the fenestrated membrane defects we observe in *ACSL* mutant flies is that they arise after patterning of the *Drosophila* eye is complete. While fenestrated membrane abnormalities have been attributed—in other mutant backgrounds—to defects in cell patterning during imaginal stages of *Drosophila* eye development, this class of patterning mutant reveals itself as a rough eye phenotype in adults, readily observed either by scanning electron microscopy or by visual examination (Coyle-Thompson and Banerjee, 1993). Importantly, we have looked for the rough eye phenotype (by visual inspection; n>100/genotype), but have yet to observe it in any of our *ACSL* mutants.

Next, we considered the lamina. Although histological studies reported here and elsewhere (Min and Benzer, 1999b) revealed gross degenerative phenotypes in *ACSL*

mutants, our TEM studies more clearly revealed the associated cellular abnormalities. Particularly notable in this regard is neuronal disorganization that appears to result from defects in axonal path finding (Fig. 2.5A,B). It is intriguing to speculate that this may be a consequence of the abnormalities in fenestrated membrane portals. Moreover, inclusions like those observed in ALD pathology as well as in other human neurodegenerative disorders including Alzheimer's and Parkinson's, appear frequently in the lamina of *bgm*^{1} *dbb*^{1} mutant animals (Fig. 2.5C,D). These may be a direct consequence of accumulating VLCFAs in mutants, as polyunsaturated VLCFAs induce formation of inclusions in a cell culture model of Parkinson's disease (Assayag et al., 2007). Consistent with this view, but leaving the inclusions unidentified, our assays with the neutral lipid stains Oil Red O and Sudan Black reveal comparable patterns in wild-type and mutant flies (data not shown). Ultrastructural studies capture lytic cell death in monopolar neurons, here visualized by cell swelling and cytoplasmic clearing (Fig. 2.5E-G; see also Fig. 2.4C,D). Finally, accumulating granular material in the proximity of dying neurons points to the possibility that monopolar neuron death is due, at least in part, to toxic cellular inclusions (Fig. 2.5H).

ACSL mutant flies recapitulate the full array of human ALD phenotypes

Having established gross anatomical phenotypes in brains of *bgm*^{1}, *dbb*^{1}, and *bgm*^{1} *dbb*^{1} adults, we sought to determine whether these defects are associated with significant changes in fatty acid levels and/or behavioral abnormalities, features that have remained statistically undocumented in previous loss-of-function studies in ALD mouse

and fly models. Fatty acid profiling by gas chromatography-mass spectrometry (GC-MS) in wild-type and *ACSL* single and double mutant flies revealed two statistically significant differences: two-fold increases of the C24:1 and C26:1 monounsaturated very long chain fatty acids in bgm^{1} dbb^{1} double mutant animals (Fig. 2.6A,B). Moreover, in tests of locomotor activity, all mutants show an inability to match wild-type activity levels as evidenced by a decrease in the average number of beam breaks per animal within the 2-hour period before and after light change. Statistically significant differences in activity levels were seen within 24 hours post-eclosion and were at or near maximal levels by 7 days post-eclosion (Fig. 2.6C). Like the morphological defects we documented above, motor defects too are reminiscent of the initial symptoms exhibited by ALD patients.

When mutations in *Drosophila* genes are associated with neurodegeneration, phenotypes oftentimes extend to life span reduction (Greenspan and Dierick, 2004), and indeed this has been previously reported for *bgm* (Min and Benzer, 1999b). In contrast to previous reports, we found that loss of *bgm* does not shorten lifespan and that loss of *dbb* only shortens life by about 10% (Fig. 2.6D). The difference between results from our *bgm* lifespan study and those reported previously is likely attributable to differences in experimental protocol. For the lifespan analyses reported here, we employed congenic recombinant inbred strains on an isogenic Canton S background. Similar to our observations, life span changes associated with a mutation in the *Drosophila* gene *Indy*, coding for a Krebs cycle intermediate transporter, were abolished upon backcrossing into a different genetic background (Toivonen et al., 2007).

Mutation of the human *bgm* homologue *Slc27a6* is associated with leukodystrophy

Dysregulation of fatty acid metabolism is associated with X-ALD, but no other human leukodystrophies have been associated with defects in this pathway (Gordon et al., 2014). In the care of a child with an MRI-based diagnosis of leukodystrophy (Fig. 2.7A,B), whole exome sequencing revealed 44 genes harboring nonsense mutations. One of these genes, *SLC27a6*, encodes a 619 amino acid dual acyl-CoA synthetase and fatty acid transporter, homologous to *Drosophila fatp* (which like *bgm and dbb* has ACSL function (supplementary material Fig. 2.2S) and has been shown to be required for neuronal heath and maintenance in the fly retina (Dourlen et al., 2012)). The proband is heterozygous for a C>T transition that leads to production of a truncated SLC27a6 protein 294 amino acids in length. This allele (designated here as $SLC27a6^{294}$) is very rare in the human population, having an overall allele frequency of 0.001% across all ethnicities (Exome Aggregation Consortium [ExAC]).

Sequence analysis of the Utah affected family indicates that both the proband and his brother inherited the mutated *Slc27a6* allele from their father and a mutated *PRRT2* allele from their mother (Fig. 7C), the latter allele being causative of an incompletely penetrant dominant form of epilepsy (Labate et al., 2012; Steinlein et al., 2012). While the proband suffers from seizures and leukodystrophy, his brother is asymptomatic for both conditions (Figure 2.7B). Comparative genotype data coupled with our observation that treatment of the proband with levetiracetam to control seizures stemmed the progression of his neurodegenerative condition leads us to speculate that in this patient, seizures are the neurodegenerative trigger. There is ample evidence for other ALD cases

where events such as epileptic seizure or traumatic brain injury have been proposed to precipitate onset of leukodystrophy symptoms in previously asymptomatic patients (Raymond et al., 2010; Vawter-Lee et al., 2015; Weller et al., 1992).

Discussion

That mutations in lipid metabolic enzymes are associated with a variety of neurometabolic diseases, including Adrenoleukodystrophy, Niemann-Pick Disease, Fabry Disease, Farber's Disease, Tay Sachs Disease, and Zellweger Syndrome, shows the nervous system to be highly sensitive to alterations in lipid homeostasis. This sensitivity is thought to result from the high metabolic demand of neurons and their support cells-- a demand not shared by cells of other organ systems. Investigators have identified the genetic basis for some of these disorders, and a number of *Drosophila* models have been described (Huang et al., 2007; Mast et al., 2011; Min and Benzer, 1999b; Nakayama et al., 2011; Phillips et al., 2008).

Here we present the first fully penetrant genetic model of ALD. Building on the Min and Benzer model (1999b), we have shown that the bgm^1 dbb^1 mutant fly exhibits gross neurodegenerative phenotypes in the optic lobe and retina. We also show that amorphic mutations in *bgm* and *dbb* lead to VLCFA accumulation and behavioral abnormalities. The consequences of the double knockout in the fly are profound, notably recapitulating essential features of human ALD that were not obvious in single mutants, thereby providing the best evidence to date of a powerful animal model of how dysregulation of fatty acid metabolism can lead to neurodegeneration. In focusing on the similarities between fly and human phenotypes associated with VLCFA accumulation,

our data suggest that ALD affects both neurons and their support cells, and that excess fatty acid accumulation is associated with widespread cell losses in the fly brain and to neuronal loss via lytic cell death. Our detection of inclusions in *bgm dbb* mutant flies augurs an attractive diagnostic tool, while the *bgm dbb* fly itself is expected to provide an effective genetic tool for identification of drugs resolving the symptoms of ALD and neurodegenerative disease associated with accumulating VLCFAs. Finally, our identification of a leukodystrophy patient harboring a mutation in a gene with ACSL activity provides a basis for examination of this gene as a susceptibility factor in ALD.

Neurodegeneration in *ACS* mutants

Albeit viable, *bgm* and *dbb* single and double mutants suffer from optic lobe and retinal degeneration. That the highest levels of neurodegeneration (both with respect to expressivity and penetrance) are observed in double mutants points to redundant functions for *bgm* and *dbb* in the *Drosophila* CNS (see Fig. 2.3). Functional overlap of *bgm* and *dbb* as long and very long chain ACSs is corroborated by elevation of very long chain fatty acids in the double mutant flies but not in the single mutants (see Fig. 2.6A,B), as well as by the overlapping long chain substrate specificities documented after heterologous expression in yeast (see Fig. 2.1D).

One of the most striking morphologic abnormalities in the bgm^{1} dbb^{1} double mutants is the loss of pigment cells (see Fig. 2.4E,F). Pigment cell organization is integral to the fly retina's lattice-like appearance, and thus loss of these cells is sufficient to account for the gross defects in ommatidial organization that we observe in mutants. Analogous disordered ommatidial phenotypes have been described in fly models of

Parkinson's neurodegenerative disease and in retinal degenerative disease (Batterham et al., 1996; Feany and Bender, 2000; Shamloula et al., 2002). In the case of the latter, retinal architectural disorder was associated with defects in pigment cells as it is also in our model. Pigment cell loss is also sufficient to account for the fenestrated membrane dysmorphologies that we observe in mutants (see Fig 2.3H, 2.4A-D), as the fenestrated membrane is formed from the feet of pigment (and cone) cells. Based on these studies, it appears that the effects of defective fatty acid metabolism in the fly retina are far-reaching, affecting cell viability in both autonomous and nonautonomous fashion. These observations contribute to the increasing recognition that pigments cells, and glial cells in general, provide critical support to the nervous system (Nave, 2010).

We also documented disarray in laminal regions of the fly CNS, and our data indicate that cell loss is central to this defect as well. Monopolar neurons in bgm^{1} dbb^{1} brains, while maintaining an intact nuclear morphology, show signs of extensive cell swelling and cytoplasmic clearing (see Fig. 2.5E-G). These ultrastructural features are characteristic of lytic cell death and of the pathological changes distinguishing lipid-rich oligodendrocyte degeneration during the initial stages of X-ALD pathogenesis (Ito et al., 2001; Tschape et al., 2002). In this regard, cerebral forms of X-ALD are associated with progressive inflammatory demyelination of white matter that is most evident in parieto-ocipital regions (Moser et al., 2007). This reaction is characterized by brain infiltration with cytotoxic T-lymphocytes and macrophages, as well as elevation of inflammatory cytokines such as TNFα and interleukin I (Powers et al., 1992). Consistent with our characterization of monopolar neuron morphology in flies, brain autopsies in ALD patients reveal loss of myelin and oligodendrocyte glial cells resulting

from cytolytic, rather than programmed cell death (Ito et al., 2001).

Accumulating VLCFAs in ACSL mutants

A long-standing debate in the ALD field centers on the question whether neurodegeneration results from an accumulation of saturated or unsaturated fatty acids. Like others, we have shown that ACSL mutation increases VLCFA levels; in *bgm dbb* double mutants, it is C24:1 and C26:1 levels that are elevated in significant fashion (see Fig 2.6A,B). This finding is consistent with our discovery that *bgm* and *dbb* have overlapping functions in the brain. With respect to human disease our results substantiate: (1) reports of elevated unsaturated fatty acids (C24:1 and C26:1) in plasma and fibroblasts from X-ALD patients (Moser et al., 1999; Moser et al., 1980), and (2) suggestions that dietary monoene therapies such as "Lorenzo's oil" are potentially inefficacious in the treatment of X-ALD (Sandhir et al., 1998). Together, these data contradict the commonly held view that accumulated saturated VLCFAs are causative of ALD. Consistent with this idea is the observation that while all individuals harboring mutant alleles of *ABCD1* exhibit increases in their circulating VLCFA levels, many do not manifest any neurodegenerative symptoms (Dubey et al., 2005; Steinberg et al., 1993).

At the cellular level, accumulating granular material in the proximity of dying neurons (see Fig 2.5F-H) points to the possibility that monopolar neuron death is due, at least in part, to toxicity of accumulated metabolic byproducts, even though Nile red and Sudan black stains suggest it is unlikely that *bgm dbb* laminal inclusions themselves represent fatty acid accumulations. Bolstering this view are published studies

including: (1) the suggestion that excess fatty acids are causative of cell death in both Leydig and adrenocortical cells in X-ALD patients (Powers and Schaumburg, 1981); (2) evidence in cultured cells that free VLCFAs induce apoptosis by activation of the pro-inflammatory TNF-mediated pathway and by formation of free radicals that induce mitochondrial damage (Feigenbaum et al., 2000; Reiser et al., 2006; Ulloth et al., 2003); and (3) the demonstration in cultured rat hippocampal cells that VLCFA challenge specifically promotes their death (Hein et al., 2008). In in vivo models, however, while metabolic abnormalities are causative of cell death, accumulated lipids serve primarily as markers of neurodegeneration (Liu et al., 2015).

At the very least, presence of fatty acid inclusions in brain tissue and the elevated levels of VLCFAs in *bgm dbb* mutant flies suggest a link between observed neuropathology and fatty acid accumulation. It should be noted that both saturated and monounsaturated VLCFAs accumulate in both plasma and fibroblasts of X-ALD patients and Zellweger patients (Powers and Moser, 1998; Sandhir et al., 1998). Similar to saturated free fatty acids, monoenolic free fatty acids show toxicity in tissue cultures causing both apoptotic and necrotic cell death (Andrade et al., 2005; Martins de Lima et al., 2006). Importantly, direct toxicity of unsaturated fatty acids was observed in neonatal ALD cells (Bachir Bioukar et al., 1994). Based on these observations, we propose that the *Drosophila bgm dbb* mutant serves as a powerful in vivo model linking fatty acid accumulation to neurodegeneration. Moreover, our data suggest that the mechanisms of neuronal cell damage observed in *Drosophila bgm dbb* mutants are similar to those associated with the first stage of X-ALD pathogenesis. The primary assault on CNS cells in X-ALD patients is believed to result from impaired homeostasis

of very long-chain fatty acids that leads to membrane instability, cell lysis and eventually to fulminant inflammatory response (Powers et al., 2005).

Drosophila models of neurodegeneration

The *Drosophila bgm dbb* mutant represents a significant addition to a growing family of fly models of neurometabolic disease. Within this group, the neurodegenerative diseases have a variety of associated abnormalities. One of the best characterized in *Drosophila* is *swiss cheese* (*sws*) (Muhlig-Versen et al., 2005). *sws* mutants suffer from a *bgm dbb*-like age dependent neurodegeneration, although holes develop more rapidly, more prominently, and across a wider expanse of the CNS than occurs in *bgm* and *dbb* mutants, and although the retina is notably spared. *sws* codes for an esterase, which when disrupted in humans leads to the neurodegenerative condition spastic paraplegia (Rainier et al., 2008). This esterase is required for hydrolyzing phosphatidylcholine, and loss-of-function mutations in either humans or flies results in an abnormal buildup of phosphatidylcholine (Fernandez-Murray and McMaster, 2007; Glynn, 2005; Muhlig-Versen et al., 2005). Thus, the etiology of degeneration for neuropathy target esterase-mediated neurodegeneration is very likely analogous to that which is proposed for Adrenoleukodystrophy-- blockage in a metabolic pathway leading to an abnormal accumulation of toxic lipids (Read et al., 2009).

More recent descriptions of *Drosophila* neurodegeneration mutants reveal those with age-dependent neurodegenerative phenotypes that like *bgm* and *dbb* target the retina. Intriguingly two of these (*dPPCS* and *fumble/dpank*) function in the CoA synthetic pathway (Bosveld et al., 2008; Siudeja et al., 2011). The *Drosophila* model of

neurometabolic disease most analogous to our *bgm/dbb* model is the *fatp* mutant. Animals harboring retinal clones homozygous for the null allele, $fatp^{k10307}$, manifest an age-dependent neurodegeneration that is characterized by disorganization of the retina and significant pigment cell loss (Dourlen et al., 2012). Taken together, the shared loss-of-function phenotypes associated with mutation of long- and very long chain-ACSs in *Drosophila* (*bgm, dbb,* and *fatp*) suggest that these genes, and the lipid metabolic pathway(s) in which they act, play an essential role in neuromaintenance, and that in the case of Fatp (as it is for Bgm and Dbb) it is the synthetase function that is necessary for CNS health and maintenance. Moreover, in light of the specific and striking shared loss-of-function *bgm, dbb,* and *fatp* phenotypes in flies, it is significant that our patient study reveals an association of *fatp/SLC27a6* loss-of-function with leukodystrophy in humans.

Association of a human ACSL with leukodystrophy

Our identification of the first leukodystrophy patient to harbor a nonsense mutation in *SLC27a6,* a gene encoding a predicted dual functioning ACSL and fatty acid transporter, is particularly compelling, as it suggests that mutations in the ACSL family of genes can be causative of neurodegeneration in humans as they are in flies. Indeed it has long been thought that ACSL mutations might be associated with ALD as ACSL levels are decreased in ALD patients (Hashmi et al., 1986; Lazo et al., 1988; Wanders et al., 1988b). The mode of inheritance of $SLC27a6^{294}$-associated neurodegenerative disease appears to be autosomal dominant, as is the case for several well-documented neurodegenerative conditions in humans (Bertram and Tanzi, 2005). The nonsense mutation that we document in our leukodystrophy patient is quite rare

(allele frequency: 0.001335), albeit more common than any of the other 41 predicted loss-of-function alleles in the ExAC database (allele frequency: 0.00007120) with the exception of one (allele frequency: 0.0022) (Exome Aggregation Consortium, 2015). The two most common alleles are almost certainly nulls: one with premature termination after AA 294; the other with a frameshift after AA 8. It is likely they each arose only once in isolated populations as each clusters in a single ethnic population database. We expect that homozygosity for loss-of-function SLC27a6 alleles results in a highly penetrant zygotic lethality as only one loss-of-function homozygote (for any allele) has been identified to date. The scarcity of mutant alleles in the human population, coupled with a paucity of diagnostic sequence data in affected individuals and an incompletely penetrant phenotype provides an explanation for our failure until now to associate *SLC27a6* with leukodystrophy. Thus, like fly *bgm* and *dbb,* and human *ABCD1*, *Slc27a6* is likely causative of an incompletely penetrant neurodegenerative phenotype, albeit in dominant fashion.

Summary

The *bgm dbb* double mutant animal has provided unique insights into the organismal (locomotor activity), tissue (neurodegeneration), and cellular (monopolar neuron and pigment cell death) requirements for long and very long chain fatty acids. Central nervous system degeneration that is associated with behavioral and biochemical abnormalities in both flies and humans underscores the power of our model for continuing to probe disease etiology as well as to initiate investigation of palliative treatment options. Moreover, in addition to revealing severe neurological involvement,

activity phenotypes can be monitored in higher throughput assays, and this opportunity underscores the feasibility of suppression screens for the identification of compounds and/or dietary regimens that alleviate the abnormalities associated with *ACS* mutation. Finally, highlighting the continuing power of simple invertebrate genetic models, this basic science study has led to our recognition of a new candidate susceptibility gene for catastrophic presentation of leukodystrophy (Vawter-Lee et al., 2015).

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Author Contributions

A.S., H.B.G., and A.L. conceived and designed the experiments and wrote the paper. A.S., H.B.G., S.S.K., and E.J.G. performed the experiments. J.L.B. identified, diagnosed, and treated the patient. All authors contributed to the analysis of data.

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Figure 2.1 *bgm* and *dbb* are duplicated genes that encode *bona fide* acyl-CoA synthetases. (A) Enzymology of VLCFA degradation in peroxisomes. When transport to the peroxisome is blocked by mutation of either the synthetase (ACSL) or the transporter (ABCD1), which is shown here as a dimer (oval pair) resident in the peroxisomal membrane, VLCFAs accumulate intracellularly (gray arrow). (B) A phylogenetic tree of the eight acyl-CoA synthetases in *D. melanogaster*. (C) Organization of the *bubblegum* and *double bubble* genes on Drosophila chromosome 2. (D) Cerulenin renders yeast auxotrophic and dependent on imported fatty acids and the activity of FAA1. Both *bgm* and *dbb* restore growth to *FAA1*-deficient yeast when myristate is added to the growth medium. Only *bgm* complements *FAA-1* in the presence of oleate.

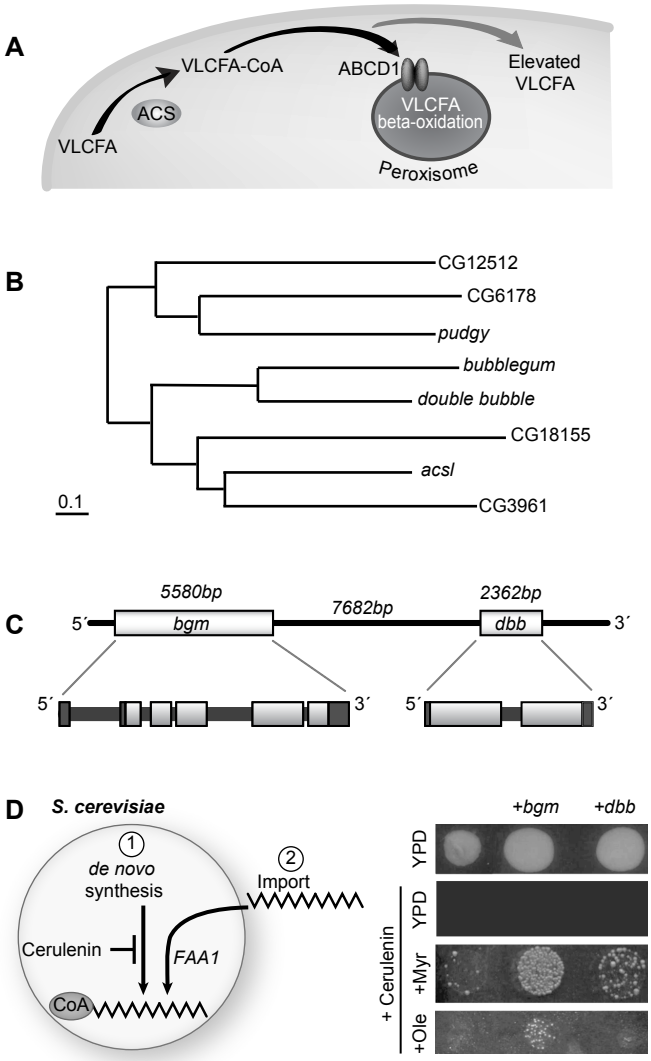

A
VLCFA
ACS
VLCFA-CoA
ABCD1
VLCFA
beta-oxidation
Peroxisome
Elevated
VLCFA
B
CG12512
CG6178
pudgy
bubblegum
double bubble
CG18155
acsl
CG3961
0.1
C
5580bp
7682bp
2362bp
5´
bgm
dbb
3´
D
S. cerevisiae
1
de novo
synthesis
2
Import
Cerulenin
FAA1
CoA
+bgm
+dbb
YPD
YPD
+ Cerulenin
+Myr
+Ole

Figure 2.2 Generation of *dbb* and *bgm dbb* mutant animals. (A) Schema showing the ends-out gene targeting strategy employed for generation of *dbb* single and *bgm dbb* double mutant animals, as well as positions of primers used for analytical PCR. (B) Verification of the putative dbb^{1} and bgm^{1} dbb^{1} alleles by PCR. M denotes the marker lane and includes bands corresponding to 10, 8, 6 and 5 kb lengths. (C) Verification of loss of *dbb* expression in dbb^{1} and bgm^{1} dbb^{1} knockout animals by RT-PCR.

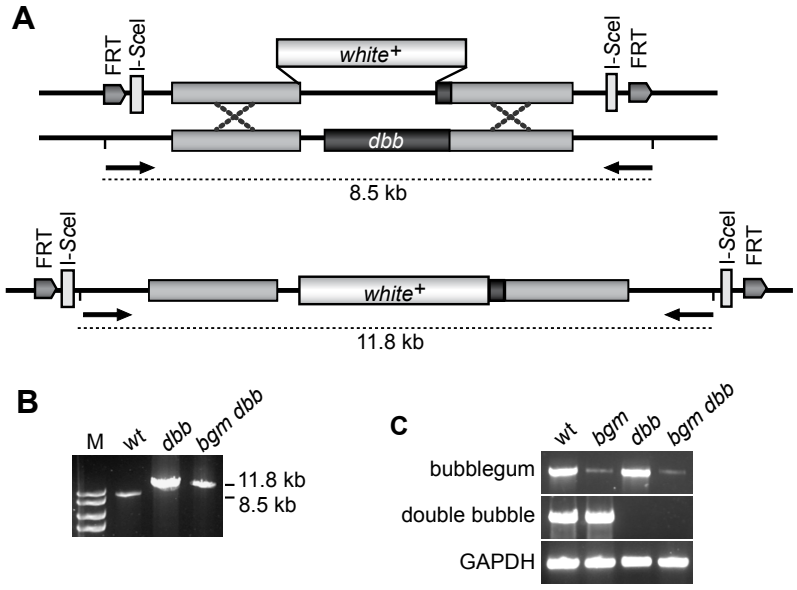

A
FRT
I-SceI
white+
dbb
I-SceI
FRT
8.5 kb
FRT
I-SceI
white+
I-SceI
FRT
11.8 kb
B
M
wt
dbb
bgm dbb
11.8 kb
8.5 kb
C
wt
bgm
dbb
bgm dbb
bubblegum
double bubble
GAPDH

Figure 2.3 Degeneration of the CNS in Acyl-CoA synthetase mutants. (A) Labeled schematization of a Drosophila brain horizontal section. (B-F) Hematoxylin-eosin stained brain sections representative of wild-type, *bgm*^{1}, *bgm*^{1}*/ Df(2L)b87e25*, *dbb*^{1}, and *bgm*^{1} *dbb*^{1} animals, respectively. In C-F retinal degeneration is marked by black arrows and laminal degeneration by white arrows. (G) Labeled schematization of a horizontal section of the Drosophila retina. (H) Magnified fenestrated membranes from animals shown in B-E. Note enlarged portals (dashed boxes, containing in the case of the *bgm dbb* double mutant displaced retinal nuclei). Degeneration in (I) lamina and (J) retina, as well as (K) defects in the fenestrated basement membrane, were quantified. For these plots, data obtained from analysis of hematoxylin-eosin brain sections were analyzed by *ANOVA* and by the Welch Two Sample t-Test. Each point represents the mean score per animal; horizontal lines represent the mean score per genotype. Statistical significance determined by ANOVA followed by student's t-test: *** $p < 0.001$, ** $p \leq 0.015$.

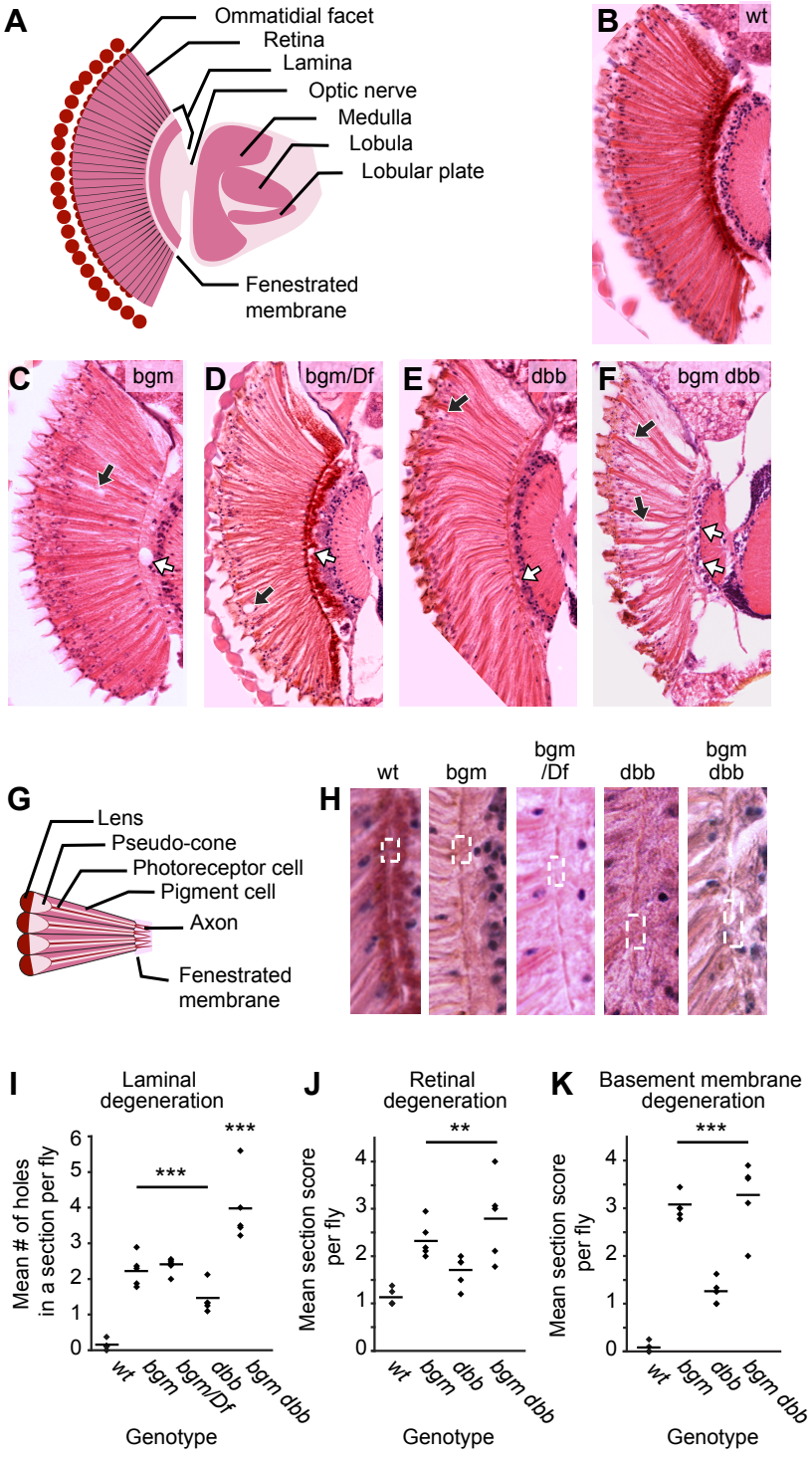

A
Ommatidial facet
Retina
Lamina
Optic nerve
Medulla
Lobula
Lobular plate
Fenestrated membrane
B
wt
C
bgm
D
bgm/Df
E
dbb
F
bgm dbb
G
Lens
Pseudo-cone
Photoreceptor cell
Pigment cell
Axon
Fenestrated membrane
H
wt
bgm
bgm /Df
dbb
bgm dbb
I
Laminal degeneration
Mean # of holes in a section per fly
***
***
wt
bgm
bgm/Df
dbb
bgm dbb
Genotype
J
Retinal degeneration
Mean section score per fly
**
wt
bgm
dbb
bgm dbb
Genotype
K
Basement membrane degeneration
Mean section score per fly
***
wt
bgm
dbb
bgm dbb
Genotype

Figure 2.4 Neuronal death and pigment cell defects in Acyl-CoA synthetase mutants. Ultrastructural analysis of heads from 18-day-old (A) wild-type, (B) *dbb*^{1}, (C) *bgm*^{1}, and (D) *bgm*^{1} *dbb*^{1} flies. A thinned fenestrated membrane is evident in comparisons of wild-type and mutant animals (boxed in panels A-D). Also, lytic death of monopolar neurons is evident in the lamina of mutant, but not wild-type animals. In A-D, monopolar neurons are indicated by arrows while R denotes the retinal domain and L denotes the laminal domain. Ultrastructural analysis of retinas from 18-day-old (E) wild-type and (F) *bgm*^{1} *dbb*^{1} flies. Loss of the ommatidial structure (outlined) in the *bgm dbb* double mutant retina is correlated with loss of secondary pigment cells; these surround each ommatidial cluster of photoreceptor neurons and in these images appear as empty white structures due to pigment loss associated with processing (Cagan and Ready, 1989; Wolff and Ready, 1991).

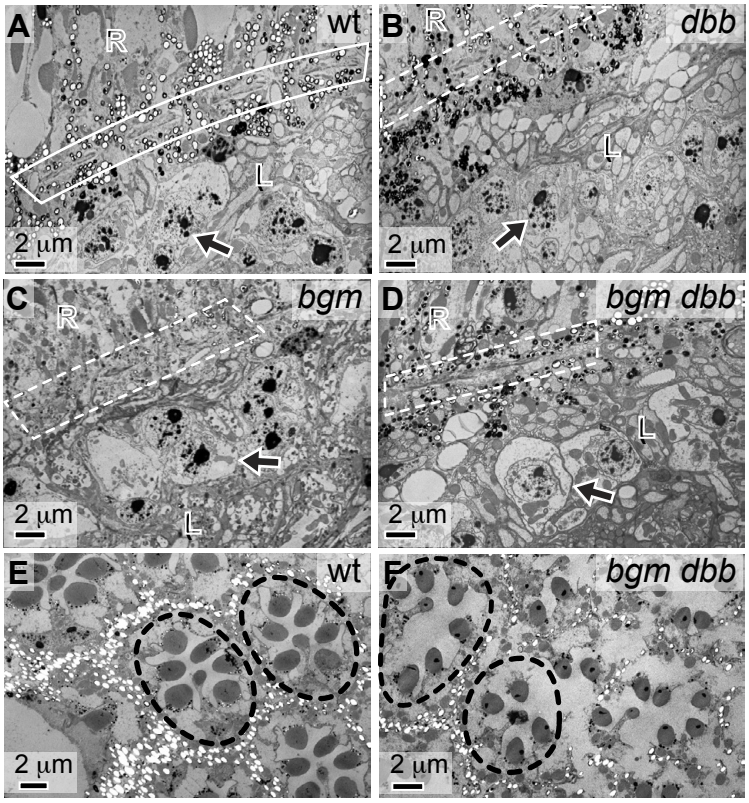

A
wt
R
L
2 μm
B
dbb
R
L
2 μm
C
bgm
R
L
2 μm
D
bgm dbb
R
L
2 μm
E
wt
2 μm
F
bgm dbb
2 μm

Figure 2.5 Neurodegeneration is associated with laminal deposits in *bgm dbb* double mutants. Ultrastructural analyses of lamina in (A) wild-type, (B) dbb^{1} and (C) bgm^{1} mutant animals reveal the lamina of mutants to be highly disorganized. Note the parallel oragnization of photoreceptor axons in wild-type brains, and their misorientation in *dbb* mutants (outlined in black in A and B). These structures are absent from *bgm* mutants in which densely stained inclusions are abundant (e.g., boxed region in C). (D) The boxed region from panel C was imaged at higher magnification. (E) In wild-type animals, monopolar neurons (outlined in white) are distinguished by their nucleolar content. In mutants (F,G), however, monopolar neurons are distinguished by cytoplasmic swelling and lytic cell death. Lysing monopolar neurons are oftentimes found in association with extracellular precipitates (arrow in F and boxed in G). (H) The precipitate from panel G was imaged at higher magnification, revealing it to be granular and thus structurally identical to previously described fatty acid precipitates.

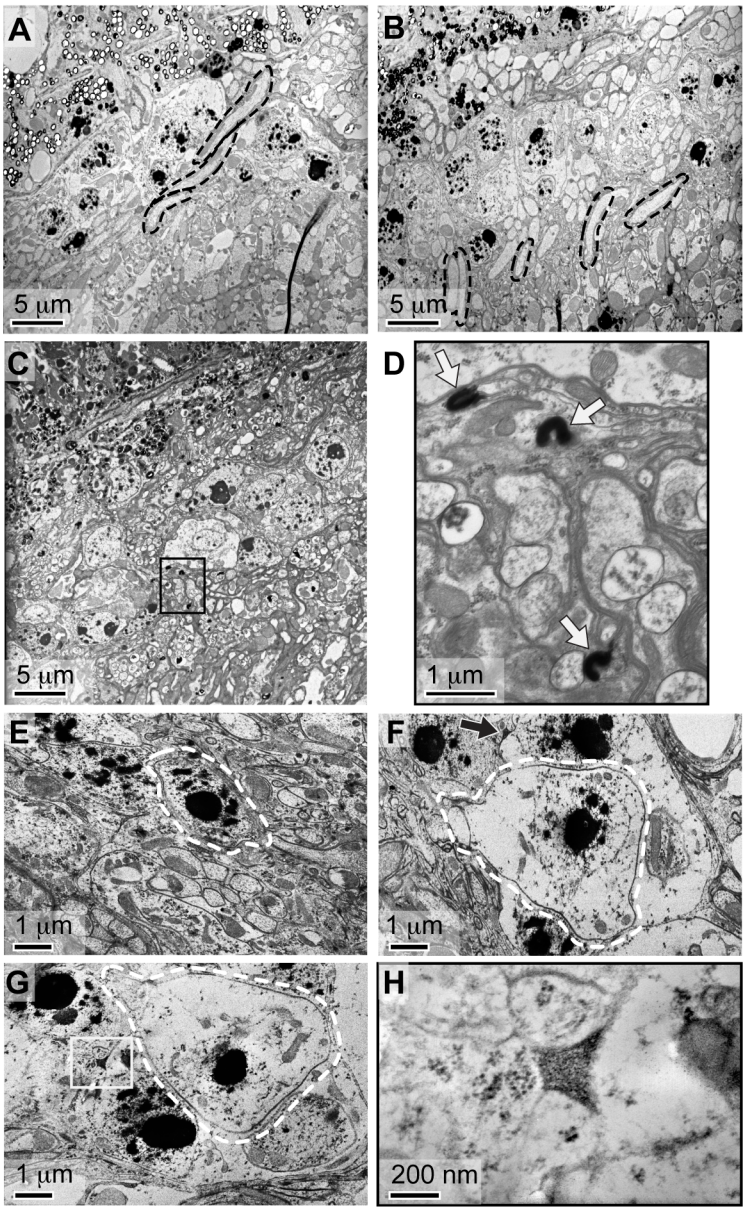

A
5 μm
B
5 μm
C
5 μm
D
1 μm
E
1 μm
F
1 μm
G
1 μm
H
200 nm

Figure 2.6. *bgm dbb* mutants recapitulate Adrenoleukodystrophy phenotypes. Gas chromatography mass spectrometry analysis (A) C24:1 and (B) C26:1 in *bgm dbb* mutant heads; *p = 0.04. (C) Locomotor activity in wild-type and mutant flies; Student's t-Test, with standard errors of the mean shown; *p < 0.05, **p < 0.01, ***p < 0.001. (C) Kaplan-Meir survival estimates for wild-type and mutant flies (n≥150/genotype).

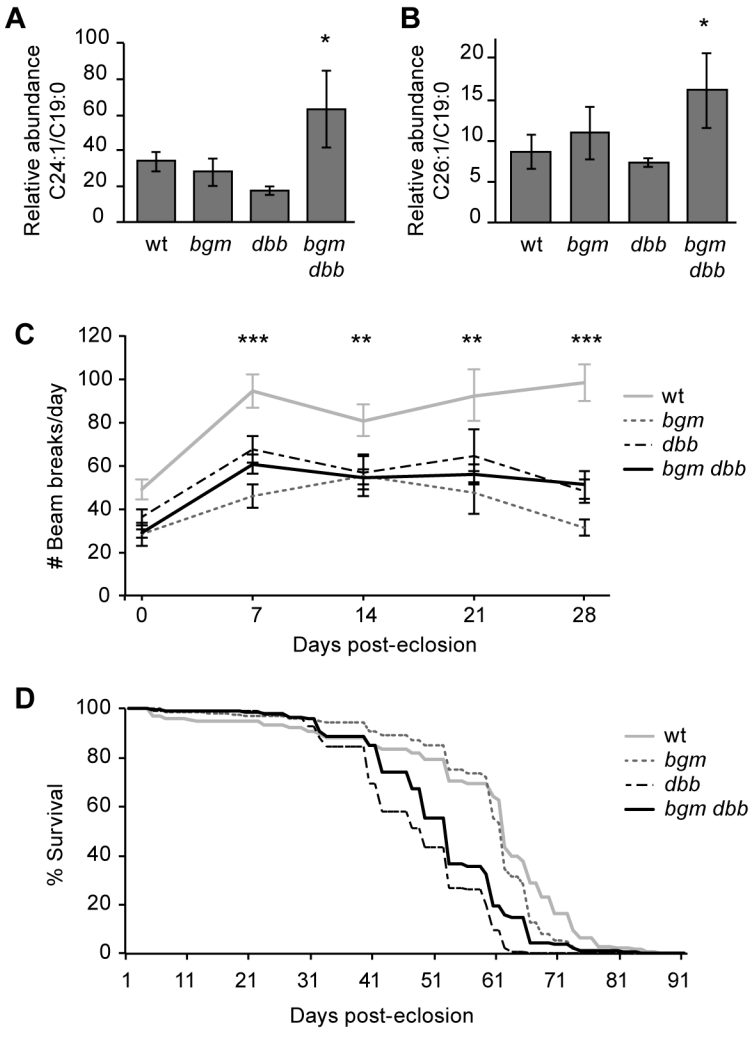

A
Relative abundance C24:1/C19:0
100
80
60
40
20
0
*
wt
bgm
dbb
bgm dbb
B
Relative abundance C26:1/C19:0
20
15
10
5
0
*
wt
bgm
dbb
bgm dbb
C
Beam breaks/day
120
100
80
60
40
20
0
***
**
**
***
0
7
14
21
28
Days post-eclosion
wt
bgm
dbb
bgm dbb
D
% Survival
100
80
60
40
20
0
1
11
21
31
41
51
61
71
81
91
Days post-eclosion
wt
bgm
dbb
bgm dbb

Figure 2.7 Identification of a leukodystrophy patient harboring a mutation in *SLC27a6.* (A) MRI scan of an 18-month-old boy presenting with epilepsy. Arrow head points to one of the expanded lateral ventricles, diagnostic of leukodystrophy; the paired structural defect is evident on the right side as well. (B) Pedigree shows family member phenotypes: PRRT2-dependent benign familial infantile epilepsy (vertical hatching); leukodystrophy (horizontal hatching). The proband is designated as #1038. (C) Sequence data for *SLC27a6* and *PRRT2*, respectively. Carriers of the *SLC27a6* mutant allele have a C>T transition; the resultant nonsense mutation leads to premature termination of the protein. Carriers of the *PRRT2* mutant allele have a C duplication at position 649; this frameshift leads to premature termination of the protein.

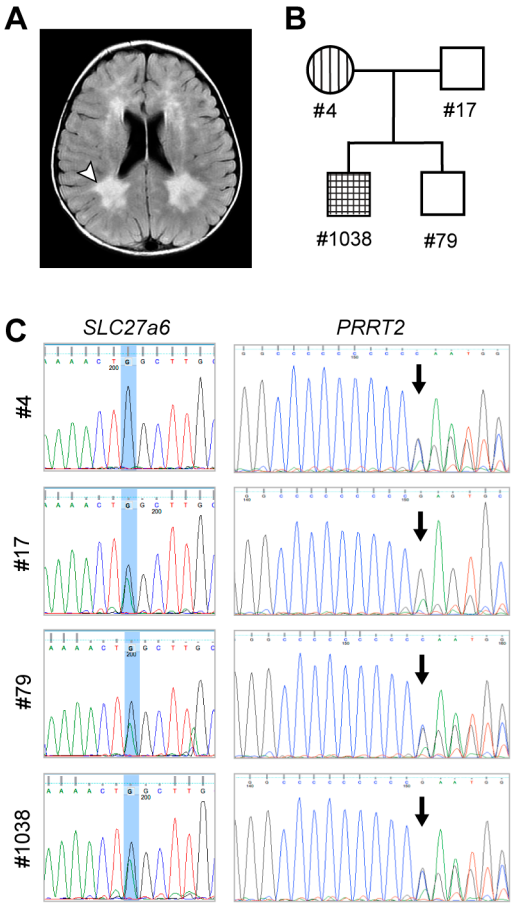

A
B
#4
#17
#1038
#79
C
SLC27a6
PRRT2
#4
#17
#79
#1038

Figure 2.S1. Basic local alignment of the *Drosophila bgm* and *dbb* genes with their human (ACSBG1 and ACSBG2) and mouse (Acsbg1 and Acsbg2) homologues.

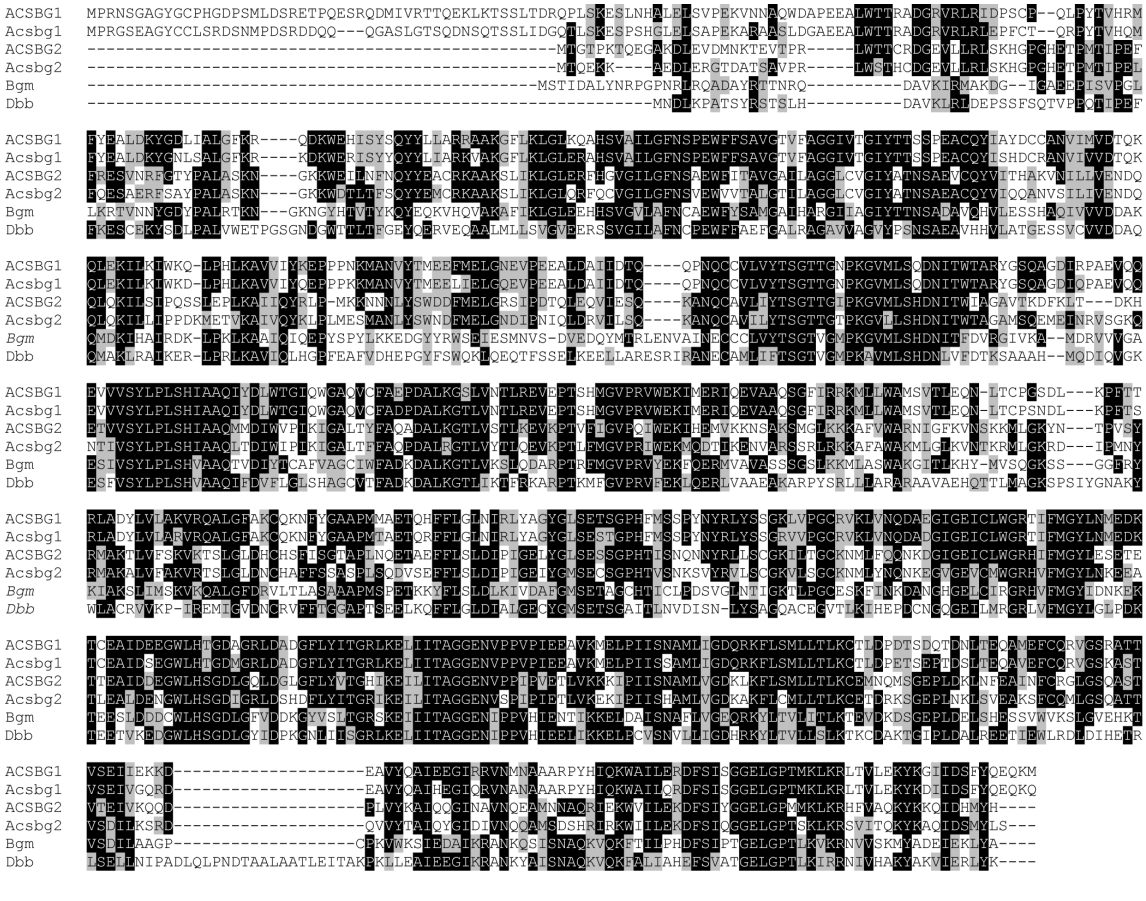
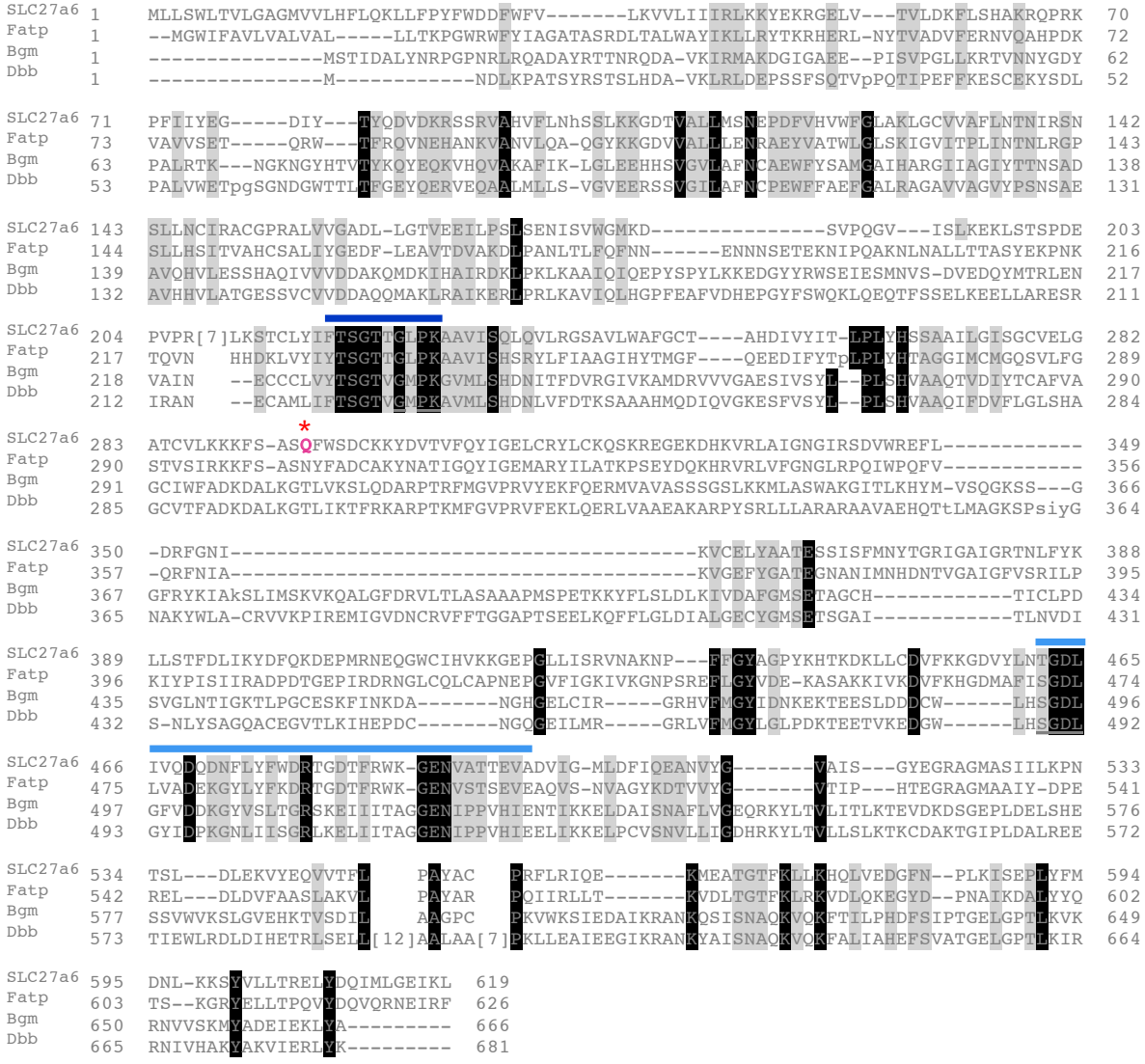

ACSBG1
Acsbg1
ACSBG2
Acsbg2
Bgm
Dbb

Figure 2.S2. COBALT alignment of human SLC27a6, and *Drosophila* Fatp, Bgm, and Dbb. Dark and light blue bars above the protein sequences delimit the two functionally conserved ACS sites: the 10 amino acid AMP binding domain and the 35 amino acid substrate specificity domain, respectively. The red asterisk demarcates the site of premature termination in the SLC27a6^{294} variant.


SLC27a6 1 MLLSWLTVLGAGMVVLHFLQKLLFPYFWDDFWFV-------LKVVLIIIRLKKYEKRGELV---TVLDKFLSHAKRQPRK 70
Fatp 1 --MGWIFAVLVALVAL-----LLTKPGWRWFYIAGATASRDLTALWAYIKLLRYTKRHERL-NYTVADVFERNVQAHPDK 72
Bgm 1 ---------------MSTIDALYNRPGPNRLRQADAYRTTNRQDA-VKIRMAKDGIGAEE--PISVPGLLKRTVNNYGDY 62
Dbb 1 ---------------M------------NDLKPATSYRSTSLHDA-VKLRLDEPSSFSQTVpPQTIPEFFKESCEKYSDL 52
SLC27a6 71 PFIIYEG-----DIY---TYQDVDKRSSRVAHVFLNhSSLKKGDTVALLMSNEPDFVHVWFGLAKLGCVVAFLNTNIRSN 142
Fatp 73 VAVVSET-----QRW---TFRQVNEHANKVANVLQA-QGYKKGDVVALLLENRAEYVATWLGLSKIGVITPLINTNLRGP 143
Bgm 63 PALRTK---NGKNGYHTVTYKQYEQKVHQVAKAFIK-LGLEEHHSVGVLAFNCAEWFYSAMGAIHARGIIAGIYTTNSAD 138
Dbb 53 PALVWETpgSGNDGWTTLTFGEYQERVEQAALMLLS-VGVEERSSVGILAFNCPEWFFAEFGALRAGAVVAGVYPSNSAE 131
SLC27a6 143 SLLNCIRACGPRALVVGADL-LGTVEEILPSLSENISVWGMKD---------------SVPQGV---ISLKEKLSTSPDE 203
Fatp 144 SLLHSITVAHCSALIYGEDF-LEAVTDVAKDLPANLTLFQFNN------ENNNSETEKNIPQAKNLNALLTTASYEKPNK 216
Bgm 139 AVQHVLESSHAQIVVVDDAKQMDKIHAIRDKLPKLKAAIQIQEPYSPYLKKEDGYYRWSEIESMNVS-DVEDQYMTRLEN 217
Dbb 132 AVHHVLATGESSVCVVDDAQQMAKLRAIKERLPRLKAVIQLHGPFEAFVDHEPGYFSWQKLQEQTFSSELKEELLARESR 211
SLC27a6 204 PVPR[7]LKSTCLYIFTSGTTGLPKAAVISQLQVLRGSAVLWAFGCT----AHDIVYIT-LPLYHSSAAILGISGCVELG 282
Fatp 217 TQVN HHDKLVYIYTSGTTGLPKAAVISHSRYLFIAAGIHYTMGF----QEEDIFYTpLPLYHTAGGIMCMGQSVLFG 289
Bgm 218 VAIN --ECCCLVYTSGTVGMPKGVMLSHDNITFDVRGIVKAMDRVVVGAESIVSYL--PLSHVAAQTVDIYTCAFVA 290
Dbb 212 IRAN --ECAMLIFTSGTVGMPKAVMLSHDNLVFDTKSAAAHMQDIQVGKESFVSYL--PLSHVAAQIFDVFLGLSHA 284
SLC27a6 283 ATCVLKKKFS-ASQFWSDCKKYDVTVFQYIGELCRYLCKQSKREGEKDHKVRLAIGNGIRSDVWREFL------------ 349
Fatp 290 STVSIRKKFS-ASNYFADCAKYNATIGQYIGEMARYILATKPSEYDQKHRVRLVFGNGLRPQIWPQFV------------ 356
Bgm 291 GCIWFADKDALKGTLVKSLQDARPTRFMGVPRVYEKFQERMVAVASSSGSLKKMLASWAKGITLKHYM-VSQGKSS---G 366
Dbb 285 GCVTFADKDALKGTLIKTFRKARPTKMFGVPRVFEKLQERLVAAEAKARPYSRLLLARARAAVAEHQTtLMAGKSPsiyG 364
SLC27a6 350 -DRFGNI---KVCELYAAIESSISFMNYTGRIGAIGRTNLFYK 388
Fatp 357 -QRFNIA---KVGEFYGATEGNANIMNHDNTVGAIGFVSRILP 395
Bgm 367 GFRYKIAkSLIMSKVKQALGFDRVLTLASAAAPMSPETKKYFLSLDLKIVDAFGMSETAGCH----------TICLPD 434
Dbb 365 NAKYWLA-CRVVKPIREMIGVDNCRVFFTGGAPTSEELKQFFLGLDIALGECYGMSETSGAI----------TLNVDI 431
SLC27a6 389 LLSTFDLIKYDFQKDEPMRNEQGWCIHVKKGEPGLLISRVNAKNP---FFGYAGPYKHTKDKLLCDVFKKGDVYLNTGDL 465
Fatp 396 KIYPISIIRADPDTGEPIRDRNGLCQLCAPNEPGVFIGKIVKGNPSREFLGYVDE-KASAKKIVKDVFKHGDMAFISGDL 474
Bgm 435 SVGLNTIGKTLPGCESKFINKDA-------NGHCELCIR-----GRHVFMGYIDNKEKTEESLDDDCW------LHSGDL 496
Dbb 432 S-NLYSAGQACEGVTLKIHEPDC-------NGQGEILMR-----GRLVFMGYLGLPDKTEETVKEDGW------LHSGDL 492
SLC27a6 466 IVQDQDNFLYFWDRTGDTFRWK-GENVATTEVADVIG-MLDFIQEANVYG-------VAIS---GYEGRAGMASIILKPN 533
Fatp 475 LVADEKGYLYFKDRTGDTFRWK-GENVSTSEVEAQVS-NVAGYKDTVVYG-------VTIP---HTEGRAGMAAIY-DPE 541
Bgm 497 GFVDDKGYVSLTGRSKEIIITAGGENIPPVHIENTIKKELDAISNAFLVGEQRKYLTVLITLKTEVDKDSGEPLDELSHE 576
Dbb 493 GYIDPKGNLIISGRLKELIITAGGENIPPVHIEELIKKELPCVSNVLLIGDHRKYLTVLLSLKTKCDAKTGIPLDALREE 572
SLC27a6 534 TSL---DLEKVYEQVVTPL PAYAC PRFLRIQE-------KMEATGTFKLLKHQLVEDGFN--PLKISEPLYFM 594
Fatp 542 REL---DLDVFAASLAKVL PAYAR PQIIRLLT-------KVDLTGTFKLRKVDLQKEGYD--PNAIKDALYYQ 602
Bgm 577 SSVWVKSLGVEHKTVSDIL AAGPC PKVWKSIEDAIKRANKQSISNAQKVQKFTILPHDFSIPTGELGPTLKVK 649
Dbb 573 TIEWLRDLDIHETRLSELL[12]AALAA[7]PKLLEAIEEGIKRANKYAISNAQKVQKFALIAHEFSVATGELGPTLKIR 664
SLC27a6 595 DNL-KKSYVLLTRELYDQIMLGEIKL 619
Fatp 603 TS--KGRYELLTPQVYDQVQRNEIRF 626
Bgm 650 RNVVSKMYADEIEKLYA--------- 666
Dbb 665 RNIVHAKYAKVIERLYK--------- 681

CHAPTER 3

REVIVAL OF DIETARY THERAPY IN A *DROSOPHILA* MODEL OF ALD SUPPORTS A REQUIREMENT OF ACSL FATTY ACID PRODUCT

Introduction

As the average age increases, so too does the risk of developing neurodegenerative disease (Hung et al., 2010). Yet, while patient populations continue to grow, the etiology of these diseases remains poorly understood, due in part to the diverse and complex nature of the molecular mechanisms surrounding neurodegeneration. Leukodystrophies represent one class of degenerative diseases that affect the white matter of the brain and have a broad range for age of onset with varying life expectancies (Gordon et al., 2014). It is estimated that a little over half of the individuals who present with the serious clinical symptom of abnormal white matter in MRI scans do not resolve to a clear causal diagnosis, exemplifying the need to understand the molecular nature of such severe defects (Bonkowsky et al., 2010; van der Knaap et al., 1999). Of the more commonly diagnosed forms of this is degenerative disease is Adrenoleukodystrophy (ALD).

At the cellular level, the only form of ALD with a defined genetic cause, results

from mutation of *ABCD1* (Moser et al., 1992). ABCD1 is a peroxisomal protein required for import of very long chain fatty acids (VLCFAs) into the lumen of the peroxisome where initial steps of b-oxidation occur. Thus, ALD disease etiology appears to be rooted in irregularities in lipid homeostasis. In this regard, ALD is not unique as many other neurodegenerative diseases are also caused by variants in genes whose proteins play key roles in lipid metabolism (acid sphingomyelinase: Niemen Pick type A OMIM#257200, alpha-galatosidase A deficiency: Fabry disease OMIM#301500, and acid ceramidase: Farber's disease OMIM#228000).

The clinical heterogeneity displayed with inheritance of the same null allele of *ABCD1* has led many to speculate genetic and/or environmental modifiers must play a role in ALD presentation. Acyl CoA synthetases represent the first, and initially favored enzyme linking the accumulation of VLCFA metabolism to ALD pathology (Lazo et al., 1988; Wanders et al., 1988). Early observations noted a biochemical defect in activation of the long and very long chain family of fatty acids in tissues of affected patients (Hashmi et al., 1986). Indeed, evidence has emerged that ABCD1 and multiple cellular ACSs physically interact and in some cases, this interaction is required for proper protein function. Yeast two-hybrid studies identify a physical interaction between mouse ABCD1 and a very long chain ACS (ACSVL1/SLC27a2) (Makkar et al., 2006). Co-immunoprecipitation studies as well as BRET (bioluminescence resonance energy transfer) assays identify ABCD1 and ACSVL5 (SLC27a1) as binding partners (Hillebrand et al., 2012). Finally, expression studies utilizing murine fibroblasts deficient for ABCD1 revealed that ACS activity is dependent on ABCD1 protein levels. This may be due to a requirement for ABCD1 to tether ACS to the peroxisomal membrane as

ACSVL1 localizes to the peroxisomal membrane (Lageweg et al., 1991; Lazo et al., 1988). Previous reports from our lab and others have documented a neurodegenerative phenotype in *Drosophila* that are homozygous for null alleles for the ACSVLs *bgm* and *dbb* (Sivachenko and Gordon et al., 2015, submitted/Chapter 2; (Min and Benzer, 1999). Both single and double mutants display age-dependent central nervous system degeneration in the optic lobe, thereby providing the most clinically relevant animal model of ALD to-date.

An increase in VLCFAs in circulating plasma and affected tissues is a key diagnostic feature of ALD (Igarashi et al., 1976), and most current models of ALD envision neurodegeneration to result from a toxic accumulation of VLCFAs within tissue leading to cell death (Berger et al., 2010). However, at least three lines of evidence do not align with this idea. First, 20% of individuals with null *ABCD1* mutations exhibit an increase in VLCFAs without any nervous system involvement (Raymond et al., 2010; Steinberg et al., 1993; Weller et al., 1992). That these individuals do not manifest neurodegenerative phenotypes despite an equivalent accumulation of VLCFA seen in affected individuals suggests that an accumulation itself is not sufficient for neurodegeneration. Additionally, VLCFA concentrations do not correlate with the degree of neurological disability (Moser, 1997). Second, multiple groups have independently generated *ABCD1* knockout mouse models. In all three reports, animals exhibit increased VLCFAs in multiple tissues and plasma (including the CNS and adrenal glands) yet do not show central nervous system phenotypes that resemble ALD (Forss-Petter et al., 1997; Fourcade et al., 2009; Kobayashi et al., 1997; Lu et al., 1997; Yamada et al., 2000). Moreover, examination of cellular morphology in ABCD1 mutants

uncovered a swollen morphology and lipid-like inclusions in the adrenal cortex, testis, and ovaries, suggesting that mutant animals do in fact suffer from a VLCFA storage disorder albeit without CNS impairment (Forss-Petter et al., 1997). In one model, aged (15 months) animals display an incompletely penetrant AMN-like phenotype in the peripheral nervous system (Pujol et al., 2002), used animals from Lu et al., 1997). Third, hematopoetic stem cell transplants in ALD patients have shown promise in halting neurodegeneration despite plasma VLCFA concentrations remaining high (Cartier et al., 2009). Taken together, these data suggest that VLCFA concentrations in ALD, while representing an important biomarker, are not causative of neurodegeneration.

In light of these data, the relevance of an increase in VLCFAs in X-ALD patients and in the *bgm dbb Drosophila* model of ALD represents a persistent question in the etiology of ALD. Another significant question in the ALD field focuses on the tissue specific requirements of ABCD1 and ACSL metabolism. At the tissue level, ALD and other leukodystrophies affect brain white matter that contains neurons as well as glia, and often a robust immune response (Powers et al., 1992). It is so far unknown whether all three of these tissue types represent cells with a primary defect due to the absence of ABCD1 or whether only some represent the primary cell type affected while other cell types represent secondary effects triggered by the initial cell death and dysfunction. Determining between these two models of tissue incorporation in degeneration is important for correct targeting of therapies for ALD. Functional tissue specific requirements of *ABCD1* or other members of the ACSL pathway have been thwarted due in part to the lack of these tools in other models, and additionally, due to a lack of a clear central nervous system phenotype in multiple mouse models of ALD (Berger and

Gartner, 2006; Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997; Pujol et al., 2002). We have shown that in the Drosophila ALD model, neurodegeneration in the CNS is profound, affecting both neurons and their support cells (Sivachenko and Gordon et al., 2015, submitted/Chapter 2) in agreement with gross tissue disruption in ALD patients (Kemp et al., 2012). Therefore, our fly model, as the only standing ALD model with central nervous system degeneration, can be uniquely used to address this question.

Building on previous reports from our lab documenting the neurodegenerative phenotype in aged *bgm* and *dbb* mutant animals we answered these long-standing questions surrounding the etiology of ALD. Here we dissect the *bgm* and *dbb* animals at the cellular and molecular levels by determining the tissue specificity of the ACSL pathway as well as the cellular defects leading to death and degeneration. These findings provide a basis for novel therapeutic approaches and strongly suggest a new proposed mechanism of neurodegenerative progression in ALD.

Materials and Methods

Drosophila stocks

Flies were raised on standard cornmeal diet at 25ºC, with 12-hour light:dark cycles unless otherwise noted. The following strains were obtained from the Bloomington *Drosophila* Stock Center and used to drive *UAS* expression: *repo-Gal4* (BL#7415), *elav-Gal4* (BL#8690), *sim-Gal4* (BL#9150), *DJ667-Gal4* (BL#8171). *tubulin-Gal4* (w1118; P{tubP-gal4}LL7}/TM3, P{Dfd-GMR-nvYFP}3 Sb1) was gifted by the Metzstein Lab (Utah). bgm^1 was gifted by Seymour Benzer. dbb^1 animals were generated as previously described (Sivachenko et al., 2015, chapter 2). RNAi lines

targeting *CG2316* and *CG2781* are from the Transgenic RNAi Project at Harvard Medical School (stocks 41984 and 50710, respectively).

Medium chain diets were prepared as previously described (Birse et al., 2010). Long chain diet was prepared as previously described (Carvalho et al., 2012). Adult males were collected within 24 hours of eclosion and maintained on all diets from within one day of eclosion to 20-22 days post-eclosion when heads were isolated and samples were blinded for plastic sectioning.

For light manipulations, males were isolated within 48 hours of eclosion and exposed to: 24 hours light, 12 hours light/dark, or 24 hours of darkness in a temperature and humidity-controlled room.

Ageing and histology

Adult Drosophila males were prepared by rinsing heads in MeOH and removing mouthparts and air sacs before fixation in 4% formaldehyde/PBS for 30 minutes at RT. Heads were dehydrated through serial MeOH/H2O washes before transferring to 1:1 MeOH:Solution A, and then equilibrated in solely Solution A at 4ºC overnight (100 ml Solution A:1gram catalyst, Electron Microscopy Sciences Immuno-Bed Infiltrate cat. no. 14260-01, 14260-06, respectively). Embedding was performed by mixing 1:20 Solutions B:A (Solution B, Electron Microscopy Sciences cat no 14260-04) and orienting heads in chuck molds for saggital sections. Plastic blocks were sectioned at 8uM thickness and stained with 0.1% Toluidine Blue to increase photoreceptor contrast. All samples were imaged at 600x using a Zeiss Axioskop 50 and Axiovision4 camera software.

Phenotype analysis and neurodegeneration scores

Three to five serial sections for each animal were blindly scored on a scale from 0-3 for qualitative disruptions in the retinal pattern where 0=no abnormalities/unaffected, 1=less affected, 2=affected, 3=strongly affected (Cao et al., 2013). Scores for each animal were then averaged within each genotype and standard error was graphed.

Transgenic generation and genetics

The coding sequence of bgm was subcloned into a pUAST vector using the following primers 5'-ATAAAGCTTATGTCCACGATAGACGCGCTC-3' and 5'-GCGGTACCGGCATATAGTTTCTCGATCTC-3'. UAS-*bgm*-FLAG plasmid was sequence verified then injected into dechorionated embryos with the attB plasmid containing the UAS-bgm-FLAG construct into the posterior end of nosΦC31;+;vk27 embryos 1-2 hours after egg lay. All surviving progeny were screened by crossing to *w*;*Dr*/TM3*Sb* for detection of germline transmission (G0 founders). Siblings of G0 founders were crossed and homozygous stocks were amplified and maintained under standard conditions. Once we generated a transgenic stock containing a single copy of UAS-*bgm* in the attP site, we next established independent lines with animals homozygous for *bgm* on the second chromosome and containing either 1) the UAS-*bgm*-FLAG transgene or 2) various tissue-specific GAL4 drivers (*reversed polarity, single minded, DJ667,* and *tubulin*). Progeny of crossing the UAS-*bgm*-FLAG line with lines containing each driver were collected within 24 hours of eclosion and aged.

Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software). In all tests, $P < 0.05$ was considered significant.

Results

Early onset of neurodegenerative disease in ACSL mutant flies

With the long-term goal of developing a more rapid and sensitive means to assess neurodegenerative phenotypes, we turned to plastic sectioning of transverse cross sections observed under light microscopy. The fly eye comprises ~800 ommatidia arranged in a highly organized rosette orientation. This regular arrangement allows us to detect even subtle disturbances of cell structure as well as finer resolution for which cell types are disturbed in mutant animals. While wild-type animals maintain a well organized retinal pattern through at least 20 days of adulthood, *bgm* and *dbb* mutant animals show a dramatic neurodegenerative phenotype (Fig. 3.1A-C). Disruption in the retinal pattern in *bgm* and *dbb* mutants was primarily due to missing glial-like pigment cells, and we rarely saw missing photoreceptors. The defects were completely penetrant in 20-day-old *bgm* animals, but incompletely penetrant in age-matched *dbb* mutants. Using this assay, we were able for the first time to identify retinal defects in both mutants as early as 5 days post-eclosion, although defects in the younger flies were more subtle in most animals (Fig. 3.1*D-F, J*). In agreement with earlier reports (Sivachenko and Gordon et al., 2015, Chapter 2; (Min and Benzer, 1999), we see no abnormalities in either wild-type or mutant animals at day 0 post-eclosion (Fig. 3.1*G-I,J*). Thus, we conclude that transverse cross sections in our system allowed sensitive detection of

neurodegeneration that can be utilized for high-throughput analysis. Defects are detectable as early as day 5 days post eclosion, and are fully penetrant at 20 days post-eclosion (Fig. 3.1 J).

The ACS/ABCD1 FA metabolic pathway functions in neurons to affect central nervous system health

To determine if Bgm function is required in neurons or glia for preventing degeneration, we utilized the UAS/GAL4 bipartite system (Brand and Perrimon, 1993) to express Bgm in neuronal or glial cells in *bgm* mutants. First, we tested whether ubiquitous expression of *bgm* rescued the defects in neuromaintenance of the retinal pattern that we see at day 20 by expressing the *bgm* transgene under the *tubulin* driver (Fig. 3.2 B). We conclude that the *bgm* transgene is functional, as we see full rescue at day 20, where we otherwise observed complete penetrance defects in maintaining retinal structure (Fig. 3.1 B). Next, we tested a tissue-specific requirement of *bgm* in neuromaintenance. To do this, we used glial (*repo*) and neuronal (*sim*) drivers, corresponding to the two tissues in which we see defects in retinal structure in *bgm* and *dbb* mutants. Surprisingly, despite bgm mutants showing primarily glial defects, glial-specific *bgm* expression was not sufficient to rescue neuromaintenance: neural specific expression, however, was sufficient for rescue (Fig. 3.2*C,D*). These data indicate *bgm* is required in neurons but not in glia for maintenance of retinal structure. Finally, expression of *bgm* primarily in muscles failed to rescue, showing specificity of expression to the retina is required for rescue (Fig. 3.2*E*).

Other ACSL pathway members are required for neuromaintenance

The neuron-specific requirements for *bgm* in *Drosophila* suggest that other ACSL pathway members implicated in ALD are also neuron specific. To test this hypothesis, we tested whether *ABCD1*—the only known gene to be causative of ALD in humans—has a neuron-specific requirement in *Drosophila*. Importantly, the requirement for the *Drosophila* homolog of human ABCD1 has never been tested (no genetic mutants). Through reciprocal BLASTp searches, we identified *CG2316* as the most closely related *Drosophila* gene to human *ABCD1*. This gene, which we will refer to as *dABCD1* is 53% identical and 71% similar to human *ABCD1*. Expression data from both FlyAtlas (Chintapalli et al., 2007) and modENCODE (Celniker et al., 2009) confirm moderate to high levels of *dABCD1* transcript expression in the adult head, correlating with a potential requirement of this gene in adult neuromaintenance (Flybase). *dABCD1* resides on the fourth chromosome and no loss of function mutations of *ABCD1* has been described. Thus, we employed RNAi to target the *dABCD1* transcript in a tissue-specific manner to test which tissues require *dABCD1* and whether the defects are similar to those caused by *bgm* loss of function. As is the case for genetic nulls of bgm and dbb, flies survive ubiquitous disruption of *dABCD1* (Fig. 3.3 A-C). *dABCD1* $^{tub>RNAi}$ adults exhibit a striking shared loss-of-function defects with *bgm* and *dbb*, including retinal holes and disrupted pigment cells upon aging (Fig. 3.3*A-C*). Targeted disruption of *dABCD1* mRNA in neuronal cells using either the *sim* or *elav* drivers resulted in retinal pattern defects indistinguishable from *bgm* mutant animals (Fig. 3.3 *D,E*). Glial expression driven under *repo*-GAL4 has no effect (Fig. 3.3 *F*). Thus, we conclude that *dABCD1*—

similar to *bgm* and *dbb*—are required in neuronal cells for correct retinal structure. With shared loss of function defects seen with three components of the ACSL pathway (*bgm, dbb, dABCD1*), we sought to establish the cellular mechanism of neurodegeneration in these animals.

ACSL enzyme fatty acid product is required for neuromaintenance

One key diagnostic feature in ALD patients is increased levels of the VLCFAs C24 and C26 in circulating plasma (Igarashi et al., 1976). These accumulated fatty acids could represent 1) a pathological accumulation of VLCFAs directly causing cellular dysfunction or 2) a byproduct of the affected pathway marking dysfunction but not participating in it. To differentiate between these scenarios, we sought to test the consequences of varying levels of available VLCFAs on neuromaintenance. We reasoned that if the neurodegeneration we observe in our ACSL-pathway mutants was due to a toxic accumulation of VLCFAs, increasing dietary intake of VLCFAs should exacerbate the neurodegeneration phenotype (Fig. 3.4 A). Therefore, we measured the extent of neurodegeneration in mutant animals fed a diet high in long chain fatty acids (which has been previously shown to impact lipid composition in *Drosophila* head tissue) compared to those fed a standard yeast diet (Carvalho et al., 2012). We found that the long chain fatty acid diet did not exacerbate retinal neurodegeneration (Fig. 3.4*B-D* vs *E-G*). These data suggest that an accumulation of long-/very long-chain fatty is unlikely to be the cause of disrupted neuromaintenance.

An alternative model is that neurodegeneration in ACLS mutants might be due to

the lack of terminally processed fatty acid products. To test this hypothesis, we fed animals a diet high in medium chain fatty acids (7% coconut oil) from day 0-20, post-eclosion. Medium chain fatty acids can be activated by other chain-length specific ACSs, making them available substrate for a four-step elongation process to provide activated long-/very long-chain fatty acids. By feeding *bgm* and *dbb* mutants excess medium chain fatty acids, we hypothesized enough long/very long-chain fatty acids could be synthesized to fulfill cellular requirements, thus bypassing the genetic block to activating LC/VLCFA products in *bgm* and *dbb* mutant animals. Indeed, we found feeding animals a diet high in medium chain fatty acids significantly reduced the retinal defects observed at day 20 post eclosion in both *bgm* and *dbb* animals (fig4*H-J* vs *E-G*)(*bgm* ND vs *bgm* MCD $p<0.01$; *dbb* ND vs *dbb* MCD $p<0.05$). These data suggest a lack of an essential fatty acid product(s) causes defects in the ability of *bgm* and *dbb* animals to maintain basal neuromaintenance.

As an extension of this idea, we asked whether artificially decreasing the cellular requirement for terminally processed fatty acid product could rescue the neurodegeneration seen in animals harboring a genetic block in L/VLCFA activation. Therefore, we used restricted application of visible light, a well-characterized environmental factor, to decrease the cellular demand for VLCFAs in *bgm* and *dbb* animals. When animals were raised from day 0 to day 20 post-eclosion in darkness compared to the normal 12 hours alternating light/dark (control), we observed a significant reduction of neurodegeneration (Fig. 3.5*B,C* vs *E,F*). We also observed a significant exacerbation of neurodegeneration in animals raised in 24-hour light conditions compared to 12 hours alternating light/dark (Fig. 3.5*H,I* vs *E,F*). Importantly,

we also observed an increase in retinal defects in wild-type animals exposed to 24 hours of light compared to 12 hours alternating light/dark (Fig. 3.5*G*). Defining the defect in wild-type animals as the amount of neurodegeneration which can be attributed solely to the environmental stress of constant light allowed us to determine that the worsening of neurodegeneration that we observe in *bgm* or *dbb* animals is more than expected if the genetic and environmental contributions to neurodegeneration were simply additive (Fig. 3.5*J*, and data not shown). Thus, we found modulating the cellular requirement for activated fatty acid products through stress modifies the defects in neuromaintenance in *bgm* and *dbb* animals, further indicating that neurodegeneration results from a lack of activated fatty acid product(s).

That *bgm* and *dbb* animals fail neuromaintenance through a lack of vital fatty acid products despite input from elongation (Fig. 3.4A) suggests that input from both the activation pathway as well as the elongation pathway is required to maintain neuromaintenance. In that case, a block in the elongation pathway should also result in neurodegeneration. We used BLAST to identify four *Drosophila* elongases; only one of these, *CG2781*, is expressed in a spatial and temporal manner based on FlyAtlas RNA seq data and modENCODE (Flybase). There are no genetic mutants described for this gene, so we tested the tissue specific requirements by using RNAi-mediated disruption. We found ubiquitous knockdown resulted in lethality before eclosion, suggesting that *CG2781* is required for viability of adult *Drosophila* (data not shown). Consistent with our previous data supporting a requirement of the ACSL pathway in neurons, we observed holes and disrupted pigment cells in the retina very similar to what we observed for *bgm* and *dbb* animals when we expressed *CG2781*-RNAi under the neuronal drivers

sim and *elav* (Fig. 3.6*B,C*). Driving *CG2781*-RNAi under the glial driver *repo* resulted in slight retinal defects that were notable, but not statistically significantly different to +/UAS-*CG2781*-RNAi animals alone (Fig. 3.6*D*)(p=0.1067). Taken together, results from diet, light and elongase studies point to a lack of terminally activated fatty acid product(s) as causative of neurodegeneration—the level of which, in wild-type animals, is likely obtained through contribution from both fatty acid elongation and activation pathways.

Discussion

ALD is a neurodegenerative disease with mechanistic roots in altered lipid metabolism. That this disease resides in a family of disorders (leukodystrophies) we incompletely understand and that there are other lipid-mediated neurodegenerative diseases (Fabry's disease, Farber's disease, Niemann Pick disease) puts our model in a unique position to provide much-needed information regarding lipid metabolism requirements in the nervous system. In order to more fully understand the etiology of ALD, and the role of ACSL pathway in neuromainenance, we first sought to determine the tissue specificity of proteins that act in this pathway. Broadly, there are two nervous system cell types—neurons and glia—and defects in both have been implicated in the pathology of leukodystrophies such as ALD (Kemp et al., 2012). Determining if the primary cellular dysfunction occurs in one or both of these cell types in each disease will assist in pinpointing the fundamental defect leading to neurodegeneration. This goal has remained elusive due to several complicating factors, one of which is the activity of other tissue types such as the immune system, which rapidly escalates white matter

degeneration thus complicating study with secondary and tertiary insults (Eichler and Van Haren, 2007). Information about tissue-specific etiology also influences treatment of these diseases, as therapeutic strategies are often influenced by which cell types are targeted (Drinkut et al., 2012). Previous observations of cell-type specificity using whole-mount immunohistochemistry directed to ALDP (the protein product encoded by *ABCD1*) in both mouse (Fouquet et al., 1997) and human tissues (Fouquet et al., 1997; Hoftberger et al., 2007) have demonstrated that this protein is expressed in at least neuronal and glial cell types. Höftenberger et al. additionally reports expression in non-neuronal human tissues. This broad expression profile seems to be conserved in *Drosophila* as *dABCD1* appears to have moderate to high expression in most tissues tested (FlyAtlas, MODENCODE, FlyBase). Given that glia and neurons share a close biological relationship, however, resolving tissue specificity to a more precise cell type requires the molecular tools of tissue-specific drivers. *Drosophila* have demonstrated exceptional utility in this regard and given our previous characterization of the *bgm* and *dbb* single and double mutant animals as additional models for ALD, we utilized our newly described models to answer such fundamental questions.

Here we present our findings that *dABCD1* and at least one other member of the ACSL pathway—*bgm*—is functionally required in neuronal cells in the adult *Drosophila* retina. Using tissue-specific expression techniques, we show that expression of *bgm* in neuronal cells, but not in glial cells is sufficient to rescue neurodegeneration in a bgm mutant background. Histologically, we initially observed that glial cells were affected prior to neuronal cells in ACSL mutants because structural aberrations disrupting these connections were the most visible and frequent. However, in reviewing other retinal

degeneration mutants, we've observed that rhabdomere disruption is often a result of a developmental defect and does not necessarily accompany all models of adult neurodegeneration (Gambis et al., 2011; Rogge et al., 1991). In models where photoreceptor degeneration is observed, ultrastructural analysis and a far later time point is often required for phenotypic analysis (Li et al., 2010; Phillips et al., 2008). We think this is reminiscent of human studies focusing on glial cell degeneration in ALD likely because these cells are more prominent in the white matter affected in the human brain (Aubourg and Dubois-Dalcq, 2000; Di Biase et al., 2004; Feigenbaum et al., 2000; Katsuragi et al., 1996; Khan et al., 1998).

Next we used a bioinformatic approach to identify the *Drosophila* homolog of mammalian *ABCD1* (called *CG2316*), which we propose to be called *dABCD1*. Ubiquitous RNAi-mediated silencing of *dABCD1* yielded viable flies with a shared loss-of-function phenotype of retinal holes seen in adult *bgm* and *dbb* mutants. The viability of adult *Drosophila* despite ubiquitous *dABCD1* knockdown mimics instances of human patients carrying loss-of-function alleles in the *ABCD1* gene and further suggests that *ABCD1* is not required for viability in either flies or humans. We next demonstrated, using tissue-specific GAL4, that this gene is also specifically required in the nervous system for neuromaintenance. Our findings demonstrate that two members of the ACSL pathway have important functions in neurons, implying disruption of this pathway is responsible for defects in neuromaintenance resulting in neurodegeneration. Given the recapitulation of the ACSL requirement for neuromaintenance in *Drosophila* and data that accumulating VLCFAs may not be sufficient for degeneration, we used our model to investigate the subcellular defect leading to cell-autonomous neurodegeneration in

mutants with disrupted ACSL metabolism.

ALD has long been hypothesized to be caused by a block in the VLCFA recycling pathway resulting in a toxic accumulation of VLCFAs. The identification of ACS mutants in *Drosophila* sharing a loss of function phenotype expanded the molecular cause of accumulated precursor to additional members of the ACSL pathway. Due in part to a clear function for this family of enzymes, the realization that a block in fatty acid activation biochemically results in 1) an accumulation of inactivated fatty acid precursor as well as 2) a lack of activated and terminally processed product uncovered the potential hypothesis that depletion of an essential fatty acid product for use within the cell could also play a role in neurodegeneration. Past studies attempting to look at the contribution of an accumulation of precursor vs. lack of product to neurodegeneration have unsuccessfully uncoupled the two components usually through complementing *ABCD1* gene function back to the cell (Shinnoh et al., 1995). The complementation of the wild-type *ABCD1* gene simultaneously clears the accumulation while providing product, maintaining the coupled nature of the metabolic reaction. We hypothesized that since the contribution of either precursor vs. product to cause neurodegeneration has not been interrogated in ALD patients, neurodegeneration may be a result of a block of the lipid metabolic pathway such that the activated lipid products are unable to be synthesized and used in the cell. Indeed, the presence of humans harboring *ABCD1* mutations as well as increased VLCFA levels, but manifesting no obvious neurodegenerative phenotype, supports this latter hypothesis (Raymond et al., 2010; Steinberg et al., 1993; Weller et al., 1992). Additionally, the dietary regime Lorenzo's oil, which normalizes VLCFA levels in patients, is ineffective at halting or preventing progress of the disease (Aubourg et al.,

1990; Moser et al., 2005; Poulos et al., 1994; van Geel et al., 1999). Our *bgm* and *dbb* mutants provide a straightforward platform to determine the requirement of an ACSL product in neuromaintenance.

We show that feeding animals a diet where the only FAs available are L/VLCFAs (overlapping with the FA chain length seen accumulating in *bgm* and *dbb* animals) is not sufficient to exacerbate neurodegeneration in *bgm* and *dbb* animals. Alternately, animals fed a high fat diet with an abundance of fatty acids that can be used as substrates in the elongase pathway (a parallel path to generate activated fatty acids) significantly rescues the neurodegenerative phenotype. Our conclusion from these studies is that an accumulation of L/VLCFA is not sufficient to cause neurodegeneration in adult *Drosophila*, and a lack of fatty acid product species may in fact represent the cellular defect resulting in neurodegeneration.

Diet studies, although useful, come with inherent complications as one attempts to track their metabolites through the maze of biological pathways. Therefore, we approached the cellular cause of neurodegeneration with the logic that if a lack of terminally processed lipid was responsible for neurodegeneration, modulating the demand for this potentially vital product might represent an environmental modifier of our phenotype. By using alternative light cycles to either increase (constant light) or decrease (constant dark) demand for terminally processed FAs demonstrates that not only that increasing the demand for product exacerbates neurodegeneration in *bgm* and *dbb* animals, but that decreasing the cellular demand alleviated neurodegeneration to near complete rescue. While these data support that a lack of product for use within the cell is the primary cellular defect resulting in neurodegeneration, future studies will be required

to further delineate which potential uses of lipids are more important to this phenotype: membrane turnover, signaling transduction, or energy metabolism.

Genetic null mutants in *fatp*, a dual fatty acid transporter and ACS, also have a retinal degeneration phenotype resulting from reduced rhodopsin signaling (Dourlen et al., 2012). The authors hypothesize that this is due to altered membrane fluidics blocking internalization of activated Rh1 protein at the plasma membrane and resulting in cell-autonomous neurotoxic activity. Because of the dual function of Fatp, this effect could not be resolved to the transport or activation function. Our results suggest neurodegeneration in *fatp* null mutants is due to the lack of ACS function, and reciprocally suggests that membrane fluidics in animals with a block in the ACSL pathway (*bgm, dbb,* or *ABCD1*) may represent the cellular use of the vital activated fatty acid product. Indeed, long- and very long- chain fatty acids are unique in that they are used as components of membranes more frequently than other chain lengths of fatty acids (Kihara, 2012; Schneiter et al., 2004). In addition, we hypothesize that our light manipulation studies may have a similar effect to other, naturally occurring environmental stresses such as trauma or epilepsy, which have previously been reported to exacerbate the clinical presentation of ALD (Raymond et al., 2010; Weller et al., 1992).

Our results also suggest a vital requirement of the elongase pathway in neuronal cells and that contribution by both the elongase and the ACSL pathways may be required to supply enough vital fatty acid product necessary for neuromaintenance. For instance, if *bgm* and *dbb* mutants represent only one arm of the path to supply a vital fatty acid product yet show a neurodegenerative phenotype, this suggests that input from both arms

of the ACSL pathway are required to supply the cell with enough activated fatty acid product. We show that, in fact, blocking the input supplying product through the elongase pathway reveals a similar retinal degeneration phenotype seen in previous genetic blocks in the activation arm of the ACSL pathway.

In conclusion, in this study, we have demonstrated that terminally processed L/VLCFA products of the ACSL pathway in neurons are required for neuromaintenance. This knowledge represents a huge leap in our understanding of ALD pathology and fundamentally shifts approaches to therapeutics by identifying neurons as a target tissue and suggesting that supplying a lacking cellular defect as opposed to removing an accumulation will yield successful therapies.

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Figure 3.1 Neurodegeneration in *bgm* and *dbb* animals. Images of plastic sections of wild-type (*A*), *bgm* (*B*), and *dbb* (*C*) adults at 20 days post-eclosion where disruption in the retinal pattern are evident in *bgm* and *dbb* mutants and appear as holes and disrupted pigment cells. Aberrations are seen in some *bgm* (E) and *dbb* (F) adults but not in wild-type adults (D) as early as 5 days post-eclosion. No detection of aberrations in wild-type, *bgm*, or *dbb* animals is seen within 24 hours of eclosion (G-I). Quantification on a neurodegeneration scale where 0=no neurodegeneration and 3=strongly affected (J). Scores were compared by one-way ANOVA with Dunnett's posttest and p-values below 0.5 were determined significant. ns=not significant, **$p<0.01$

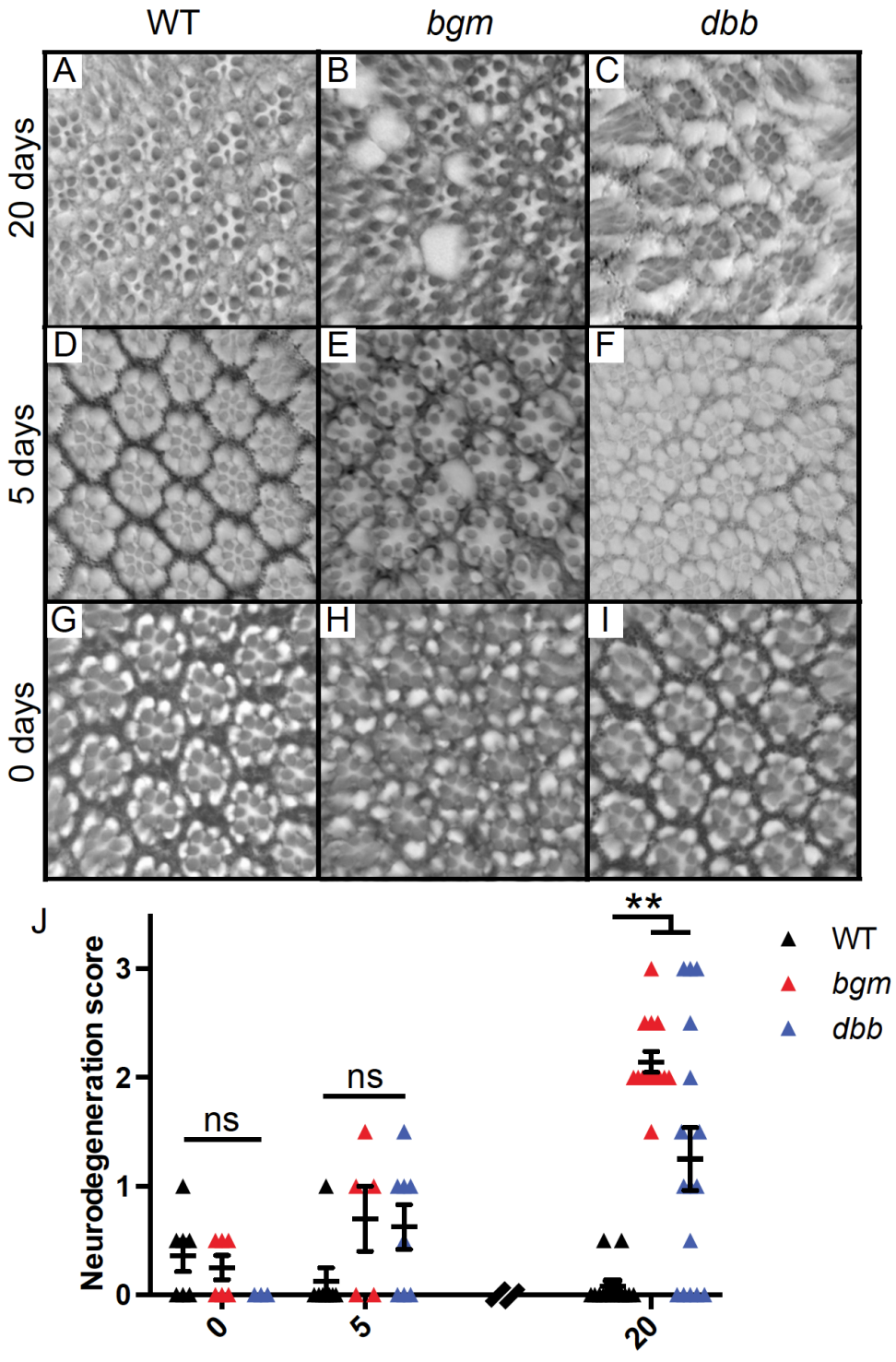
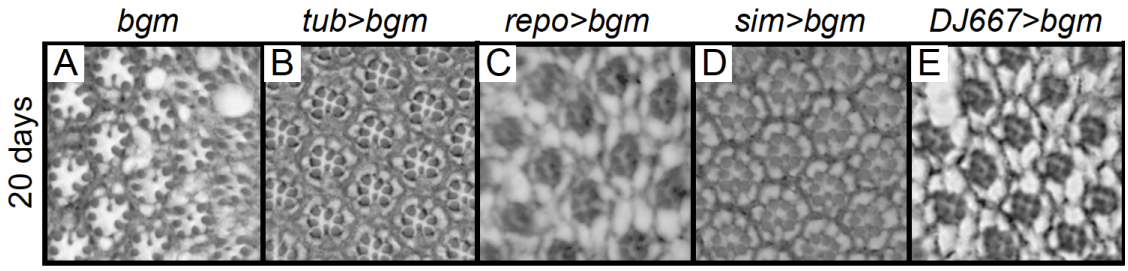

WT
bgm
dbb
A
B
C
D
E
F
G
H
I
20 days
5 days
0 days
J
Neurodegeneration score
0
1
2
3
ns
ns
**
0
5
20
WT
bgm
dbb

Figure 3.2 Expression of *bgm* in adult retinal neurons is sufficient to rescue neuromaintenance in *bgm* animals. Representative cross-section of a *bgm* mutant at day 20 post-eclosion (A). Ubiquitous expression of an inducible *bgm* construct with *tubulin*-GAL4 rescues neurodegeneration in a *bgm* mutant background (B). Driving expression in glial cells (*repo*) does not result in rescue (C); however the neuronal driver single-minded (*sim*) does rescue (D). DJ667 was used as a negative control as it is specifically expressed in flight muscles and fails to rescue (E). Quantification of neurodegenerative phenotype (F). Scores were compared by one-way ANOVA with Dunnett's posttest and p-values below 0.5 were determined significant. ***$p<0.001$.


bgm
tub>bgm
repo>bgm
sim>bgm
DJ667>bgm
A
B
C
D
E
20 days

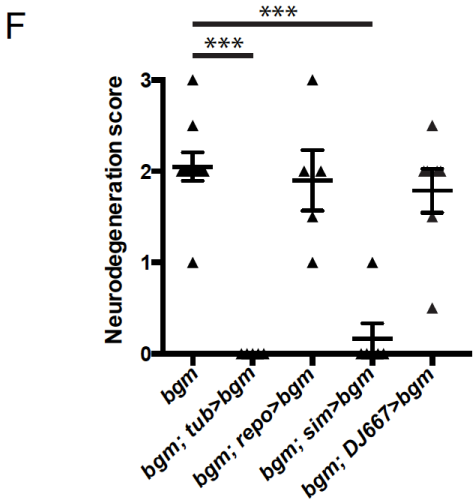

F
***
***
Neurodegeneration score
3
2
1
0
bgm
bgm; tub>bgm
bgm; repo>bgm
bgm; sim>bgm
bgm; DJ667>bgm

Figure 3.3 The *Drosophila* homolog of *ABCD1* is required for neuromaintenance. Retinal cross sections of a *tubulin*-GAL4/+ (A), and driver-less UAS-*CG2316*-RNAi control animals (B). Ubiquitous knockdown of *dABCD1* results in a shared loss-of-function phenotype with *bgm* and *dbb* mutants (C). Neuronal knock-down of *dABCD1* (D, E) but not glial knock-down (F) recapitulates neurodegeneration. Quantification of neurodegenerative phenotype A-F (G). Scores were compared by one-way ANOVA with Dunnett's posttest and p-values below 0.5 were determined significant. ***$p<0.001$

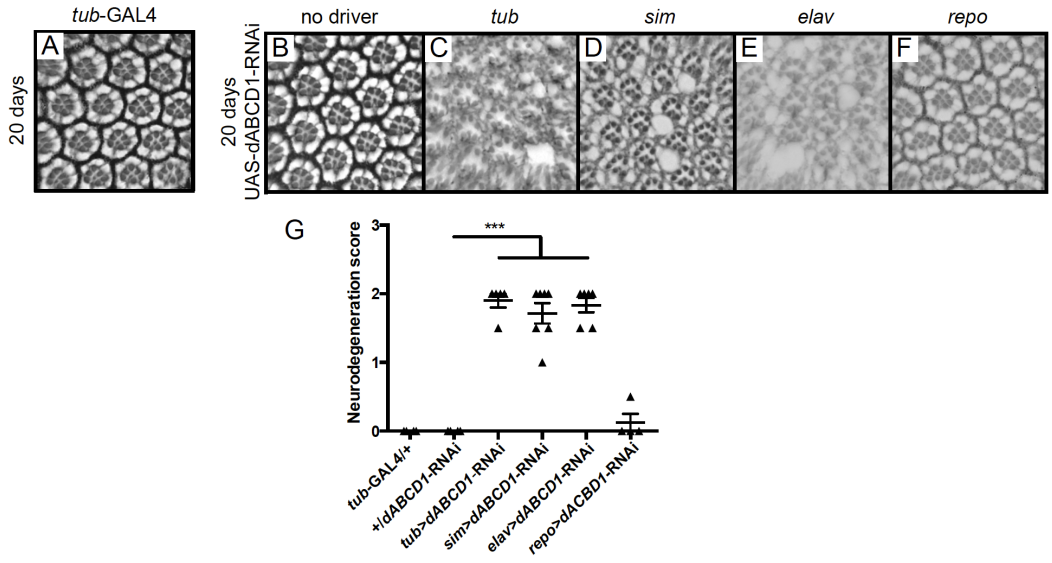
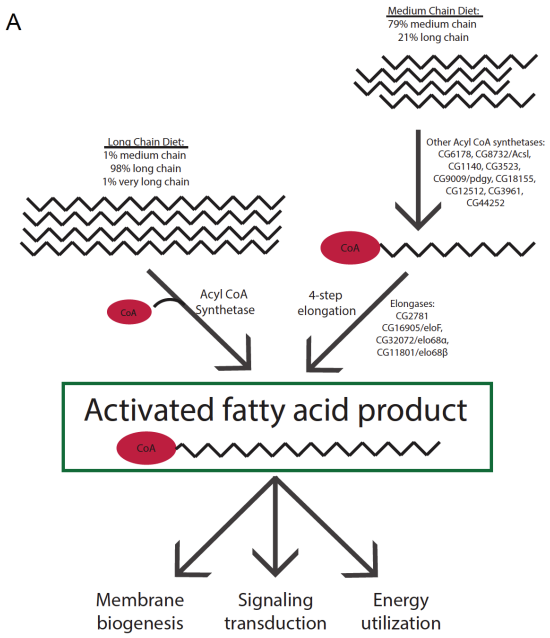
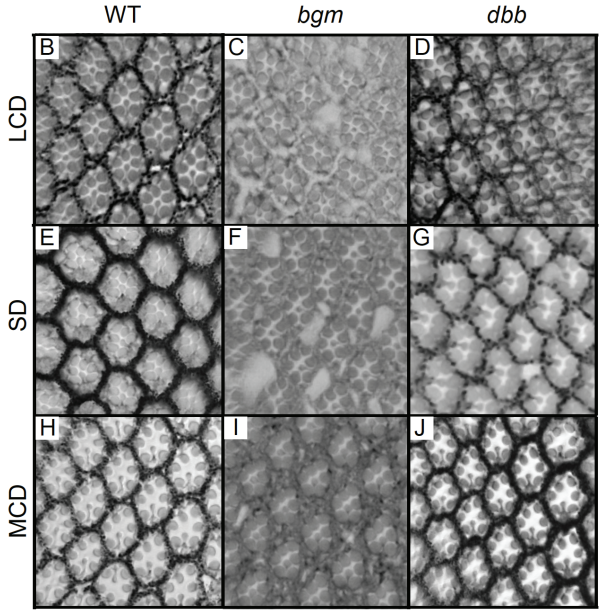

tub-GAL4
no driver
tub
sim
elav
repo
20 days
20 days
UAS-dABCD1-RNAi
A
B
C
D
E
F
G
Neurodegeneration score
***
tub-GAL4/+
+/dABCD1-RNAi
tub>dABCD1-RNAi
sim>dABCD1-RNAi
elav>dABCD1-RNAi
repo>dACBD1-RNAi

Figure 3.4 Neurodegeneration in *bgm* and *dbb* animals is caused by a lack of activated fatty acids. Two pathways converge to produce activated long-/very long-chain fatty acid products within cells (A). Dietary supplementation of either long or medium chain lengths of fatty acids were used to test if an accumulation of fatty acids in *bgm* and *dbb* animals is causative of neurodegeneration. Animals fed a diet where the only fatty acids are long (LD) did not experience a worsening of neurodegeneration (B-D vs E-G). Animals fed an excess of medium chain fatty acids (MD) as substrate for the elongase pathway showed an alleviation of neurodegeneration (H-J vs E-G). Quantification of neurodegeneration in animals on a normal diet versus a long chain diet (K) and a medium chain diet (L). Scores were compared by one-way ANOVA with Dunnett's posttest and p-values below 0.5 were determined significant. ns=not significant **p<0.01, *p<0.05


A
Medium Chain Diet:
79% medium chain
21% long chain
Long Chain Diet:
1% medium chain
98% long chain
1% very long chain
Other Acyl CoA synthetases:
CG6178, CG8732/Acsl,
CG1140, CG3523,
CG9009/pdgy, CG18155,
CG12512, CG3961,
CG44252
CoA
CoA
Acyl CoA
Synthetase
4-step
elongation
Elongases:
CG2781
CG16905/eloF,
CG32072/elo68α,
CG11801/elo68β
Activated fatty acid product
CoA
Membrane
biogenesis
Signaling
transduction
Energy
utilization


WT
bgm
dbb
LCD
SD
MCD
B
C
D
E
F
G
H
I
J

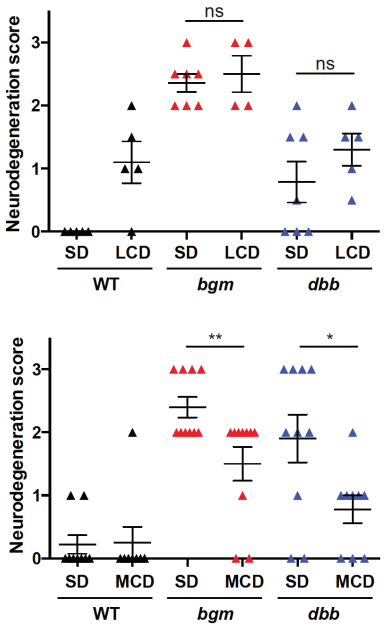
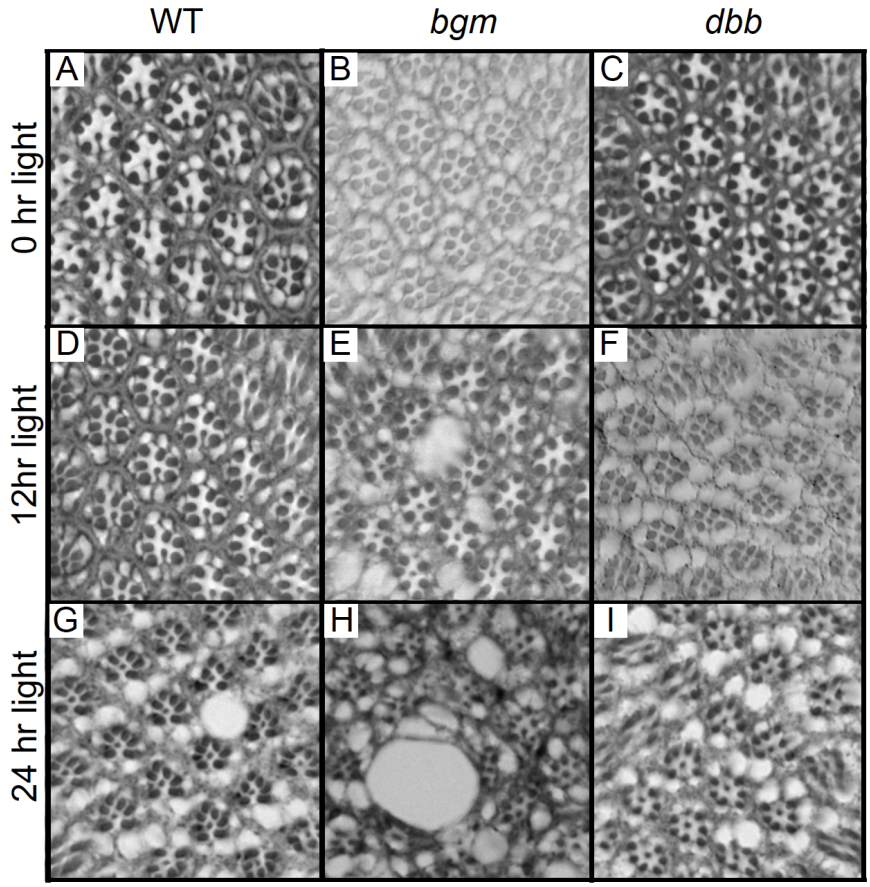

K
Neurodegeneration score
ns
ns
SD
LCD
SD
LCD
SD
LCD
WT
bgm
dbb
L
Neurodegeneration score
**
*
SD
MCD
SD
MCD
SD
MCD
WT
bgm
dbb

Figure 3.5 Modulation of the demand for activated fatty acids modifies *bgm* and *dbb* neurodegeneration. Cross sections of animals exposed to decreased light at zero hours of light/day (A-C), standard light at 12 hours of light/day (D-F), and increased light at 24 hours of light/day (G-I). Quantification of neurodegenerative phenotype (J). Scores were compared by one-way ANOVA with Dunnett's posttest and p-values below 0.5 were determined significant. ***$p<0.001$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A
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C
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G
H
I
0 hr light
12hr light
24 hr light

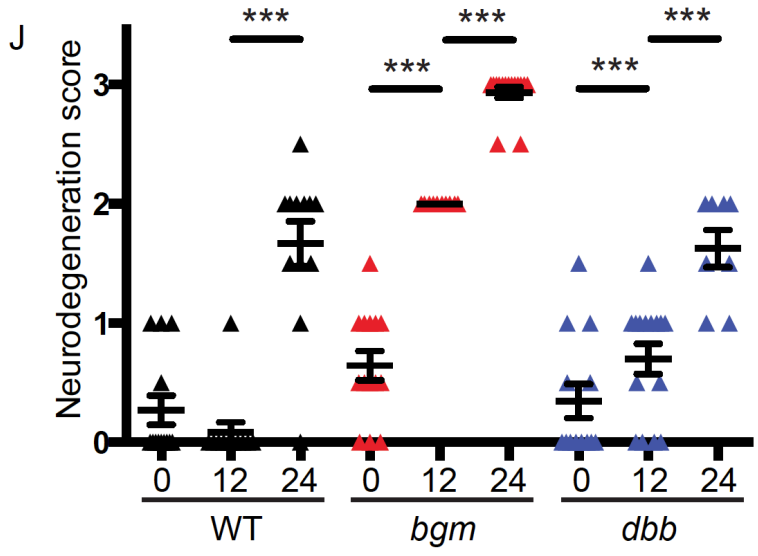
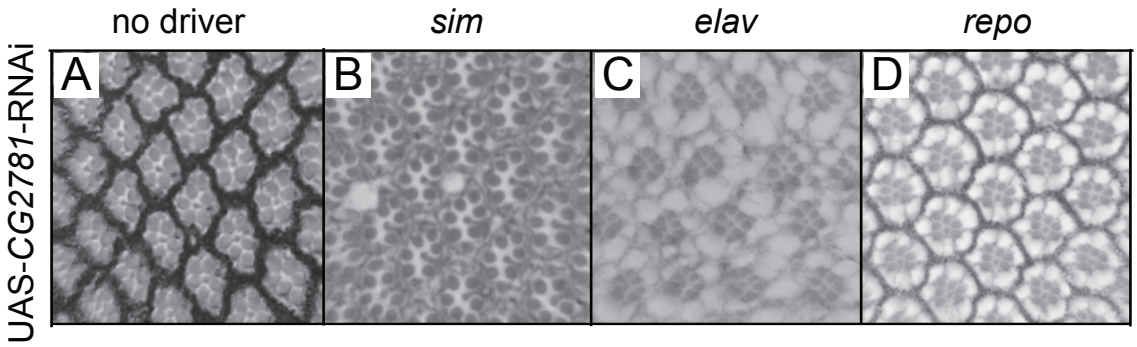
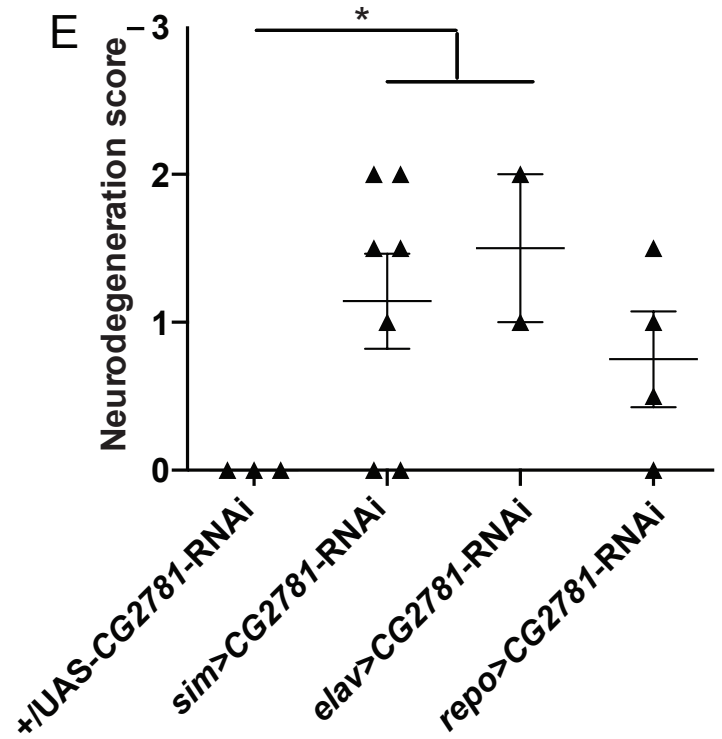

J
Neurodegeneration score
***
***
***
***
***
***
0
1
2
3
0
12
24
0
12
24
0
12
24
WT
bgm
dbb

Figure 3.6 Decreasing the amount of fatty acid product by knocking down input through the elongase pathway also results in neurodegeneration. Expression of RNAi targeting the *Drosophila* elongase *CG2781* in neuronal cells results in a shared loss of function phenotype with *bgm* and *dbb* animals (B,C). Quantification of neurodegenerative phenotype (E). Scores were compared by one-way ANOVA with Dunnett's posttest and p-values below 0.5 were determined significant. *$p<0.05$


no driver
sim
elav
repo
UAS-CG2781-RNAi
A
B
C
D


E
Neurodegeneration score
0
1
2
3
*
+/UAS-CG2781-RNAi
sim>CG2781-RNAi
elav>CG2781-RNAi
repo>CG2781-RNAi

CHAPTER 4

ADDITIONAL INSIGHTS INTO *BGM* AND *DBB* NEURODEGENERATION

Introduction

This chapter describes three experiments that are beyond the scope of primary dissertation research. Results from these experiments were of limited use as they are focused on narrow hypotheses. Reported here, they serve two purposes: to make this dissertation comprehensive of the work I undertook in the Letsou lab, and to serve as a potential launching pad for future lab members. Thus, these three experiments represent mere observations that should thoroughly be tested, but nonetheless considered as the model for the mechanism of neurodegeneration in *bgm* and *dbb* mutants evolves.

First, data in previous chapters of this dissertation have suggested a requirement in neuronal cells for a terminally processed activated fatty acid product in neuromaintenance in adult *Drosophila*. However, the specific identity and cellular use of this fatty acid are so far unknown. In this chapter, I will provide preliminary evidence pointing to one cellular pathway in which the terminally processed fatty acid functions. These studies have a particular relevance to therapeutic strategies as well as understanding the biological requirements of the nervous system.

The simplest explanation for the requirement of ASCL pathway members in neuromaintence would predict expression of *bgm* and *dbb* in neuronal tissue. Indeed, in Chapter 3, I show a requirement of *bgm* in neuronal tissue for maintenance of retinal structure in adult *Drosophila*. In this chapter, I determined *bgm* and *dbb* are expressed in adult *Drosophila* retinal tissue. However, resolution to expression in either glial or neuronal cells is difficult to resolve given the methods used. Nonetheless, both *bgm* and *dbb* appear to be expressed in tissues in the retina, possibly consistent with a neuronal requirement of these genes.

Second, lipids play three major roles within cells: as components of membranes, as energy substrates, and as mediators in signaling cascades (Schmitt et al., 2014). All of these roles need to be fulfilled for all cells. However, it is unknown if nervous tissue possesses a particular sensitivity to some of these uses. Without fully answering this question, there are examples of neurodegenerative models that result from a defect in some of these particular pathways discussed below.

Energy utilization represents a well-known tissue-specific cellular requirement. In addition to tissue specific requirements of absolute energy consumption (Wang et al., 2010), tissues can also show a specific requirement for energy substrates. With regard to nervous tissue, it has long been known that glucose is the sole energy substrate used by the brain (Siegel, 1999). Initial studies of this kind were done by measuring arteriovenous differences for various metabolites (Greger and Windhorst, 1996) and bolstered, still early on, by determining the respiratory quotient of brain tissues to be equal to 1.0—the value rendered when glucose is solely used as a substrate (Dickens, 1936). Although it is widely accepted that the brain only utilizes glucose for energy, it is

important to note that finer resolution of neuronal and glial energy utilization and intra-tissue signaling is an ongoing area of research with continued capacity to inform neurometabolic disease (Belanger et al., 2011).

With regard to this dissertation, due to the known use of glucose as the energy substrate used by neuronal tissues, it is unlikely that terminally processed fatty acid product(s) deficient in *bgm* and *dbb* single and double mutant animals impacts energy production. However, the possibility remains that mitochondria still lie at the heart of neurodegeneration in these animals, as mitochondria not only represent a hub of energy metabolism but also important signaling molecules. In this chapter, I report initial investigations into the role mitochondria play in ASCL-mediated neurodegeneration. I test whether mitochondria play a cellular role in neurodegeneration by testing whether mitochondrial stress in the form of hyperoxic conditions exacerbates *bgm* and *dbb* single and double mutant animals to defects in neuromaintenance.

Finally, cellular membranes are increasingly being recognized for their complex composition and their ability to affect signaling. For this reason, it is often difficult to parse membrane-signaling defects, in particular when signaling molecules are components of cellular membranes, such as phospholipase C (PLC). PLC is a conserved, core molecule in the cellular response to light. Exposure to light first induces a conformational change in the photopigment Rhodopsin that in turn activates a heterotrimeric G protein. Release of the Gα subunit leads to activation of PLC, an integral membrane protein that cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers and releases a proton, soluble inositol 1,4,5-triphosphate (IP_3), and diacylglycerol (DAG). DAG and IP_3 both independently

activate protein kinase C (DAG directly, IP_3 via release of intracellular Ca2+ pools), and this further transduces the signal within the cell. PLC activation also results in activation of transient receptor potential channels (TRP channels) that depolarize the photoreceptor cell and propagate activation through retinal tissue. PLC is required for detection of light in *Drosophila* and loss-of-function alleles of *norpA* (the gene encoding PLC in *Drosophila*) represent some of the first identified light-sensitive mutants (Cosens and Manning, 1969). Highlighting the intricate relationship between this important signaling molecule and plasma membrane architecture is the recent discovery that PLC activates TRP channels through rapid degradation of PIP_2 into DAG, the latter of which occupies less space in the membrane than what PIP_2 occupies. The rapid decrease in occupied membrane space when PIP2 is converted to DAG leads to contraction of the plasma membrane and consequent mechanical activation of TRP channel activity (Hardie and Franze, 2012). Although no mutations of human *PLC* have been identified (potentially due to its participation in many other signaling pathways), constitutively active variants of Rhodopsin are the most common cause of Autosomal Dominant Retinitis Pigmentosa which leads to overactive rhodopsin signaling resulting in cytotoxicity and neurodegeneration (Mendes et al., 2005).

Drosophila harboring mutant clones of a null allele of *fatp* ($fatp^{k10307}$) suffer from neurodegeneration. The postulated mechanism leading to neurodegeneration is cell-autonomous cytotoxicity as Rh1 is reported to accumulate in *fatp* mutants and dominant negative alleles of *rhodopsin 1* as well as *arrestin* fully rescue degeneration in these animals (Dourlen et al., 2012). Due to the dual nature of Fatp as both a fatty acid transport protein and an acyl CoA synthetase, however, the cytotoxic defect could not be

narrowed to a single function of Fatp. In this chapter, I present preliminary evidence that rhodopsin accumulates in *bgm* and *dbb* mutants. However, different experiments are required to determine if this is a secondary effect of dysfunctional cells or a primary cause of dysfunction.

Materials and Methods

In situ hybridizations

In situ hybridizations were performed on cryosections of whole animal adults. Briefly, adult males were embedded in 7.5% gelatin, 15% glucose, flash frozen in liquid nitrogen and sectioned at a thickness of 10 μM. After sectioning, samples were immediately transferred to dry ice chambers to preserve RNA. *bgm* and *dbb* probes were made using the following primers: *bgmF* 5'-CATTAGGCGGGTTAAATTCCCG-3' *bgmR* 5'-GTCGACGTTAGAACGCGGCTAC-3', *dbbF* 5'-TTGTAAAACGACGGCCAGTGA-3' *dbbR* 5'-GGAAACAGCTATGACCATGATTACGC-3' incorporating digoxygenin. Sequences are present in WT animals and either not present in mutants or present at very low levels (*bgm* mutant retains ~1% that of wild type). Hybridizations were performed as previously described (Wülbeck and Helfirch-Forster, 2007) and modified slightly for cryosections instead of whole mount tissue. Samples were imaged using a Zeiss Axioskop 50 equipped with an Axiovision4 camera.

Western blots

Eclosed males were collected over a 24-hour period and aged for the times indicated. An average of 10 heads were isolated on ice in Laemmli buffer (10%glycerol, pH6.8 0.5M Tris, 10% SDS, 1% bromophenol blue, 1% β-mercaptoethanol, 100mM DTT) at a concentration of 1 head/3ul buffer, homogenized, and centrifuged for 30 minutes at 12,000xg at room temperature. Supernatant was collected and boiled for 5 minutes before storage at -20ºC or loading on a 12% acrylamide gel and transferred onto Immobilon-FL (Millipore). Anti-Rh1 (1/1000, 4C5, DSHB) and anti-β-actin (1/1000, Novus Biologicals) were incubated overnight at 4ºC before the appropriate secondary anti-mouse and anti-rabbit antibodies (1/10000, IRDye 680T and 800CW, LI-COR) were then incubated for 1 hour at room temperature. Protein bands were detected and quantified by infrared using a LI-COR Odyssey Infrared Imager and Odyssey software. Sample isolation was performed consistently between the hours of 12p-5pm to minimize a bias of Rh1 expression before exposure to daily light.

Hyperoxic chamber

Male flies were collected within 24 hours of eclosion, transferred to vials at a density of approximately 20 flies/vial, and housed in a humidified hyperoxic chamber maintained at 88% O2. Dead animals were assayed daily and a vial change was performed at day 10 with vials preacclimated to hyperoxia.

Results

To test the hypothesis that the ACSL pathway is required cell autonomously for neuromaintenance, I assayed *bgm* and *dbb* expression in the adult *Drosophila* head. *bgm* expression was restricted to minimal retinal staining, a defined stripe at the fenestrated membrane, and peripheral staining around the medulla (Fig. 4.1A-B). *dbb*, on the other hand, is expressed more broadly across the retina and central brain (Fig. 4.1C-D). Using this method, it is difficult to determine which nervous tissue (neuronal or glial-like pigment cells) expresses *bgm*. However, the rescue experiments performed in Chapter 3 suggest neuronal expression. It is likely that both neuronal and glial cells express *dbb*.

Mitochondria play a role in mediating cell death and swollen mitochondria were observed in electron microscopy images in *bgm dbb* mutant animals (Sivachenko, unpublished). To determine whether mitochondria play a central role in neurodegeneration in *bgm* and *dbb* single and double mutants, we raised animals in a hyperoxic environment and assayed whether mitochondrial stress exacerbates the slight but significant lifespan defect in mutant animals (Freeman and Crapo, 1981). We saw an equivalent effect on the lifespan in wild type and all mutant animals (Fig. 4.2). Thus it is unlikely that mitochondria likely do not play a central role in neuronal dysfunction leading to cell death in *ACSL* mutants. It should be acknowledged that hyperoxic stress represents one among many methods to test mitochondrial involvement and results from this study alone do not disprove the hypothesis. These studies should be confirmed with finer tests of mitochondrial function discussed later in this chapter.

Lastly, given the proposed role of Fatp (another ACS in *Drosophila)* in neuromaintenance, we examined whether our *ACSL* mutants similarly function through

rhodopsin to effect neurodegeneration. It has been proposed that in *fatp* mutants, activated rhodopsin cannot be internalized for recycling thereby leading to excitatory cell death in neuronal cells of the adult retina (Dourlen et al., 2012). As a first step to investigating this possibility *in* bgm and *dbb* mutants, we performed western blot analysis to assess if Rh1 is accumulating in *bgm* and *dbb* mutants as an initial test of a shared mechanistic pathway to neurodegeneration. Dourlen et al. report a two-fold accumulation of Rh1 via western blot in pooled animals aged from day 1-11 post-eclosion. Analysis of our animals over a longer and more precise time course showed incomplete penetrance of accumulation in *bgm* animals, pointing to Rhodopsin either as an effector of disease as predicted to be the case for *fatp*, or as a marker of dying cells. We see no differences between wild type and mutant animals at 2 days post eclosion (Fig. 4.3A). At day 8, day 10 and days 22-25, some *bgm* samples appear to contain more rhodopsin than wild type animals. A time point in the middle of this span (day 12), however, does not appear to have any samples with increased rhodopsin expression. Additionally, we observed increases much higher than two-fold resulting in an overall increase in the range of values observed. Of note, at only the day 22-25 time point do *dbb* animals possibly contain more rhodopsin that wild type. We conclude that at present, these results are inconclusive. As mentioned, even if rhodopsin is accumulating, rescue studies would be needed to discriminate between its role as an effector or marker of disease.

Discussion

Mitochondria play well-characterized roles in signaling, especially relating to cell death (Liu et al., 1996). Our hyperoxic study suggests mitochondria do not play a role in cell death in *bgm* and *dbb* single and double mutant neurodegeneration. This finding is consistent with conclusions derived in studies of ABCD1 protein-deficient mice where despite accumulation of VLCFAs in whole muscle homogenates mitochondrial function remains intact (Oezen et al., 2005). Due to the pleiotropic nature of reactive oxygen species acting not only on mitochondrial proteins, but also on all molecules present in an organism, my experiment was poorly focused. A better strategy might be inclusion of p35 in neuronal tissue to determine if autonomous inhibition of cell death rescues neurodegeneration (Hay et al., 1994).

Fatty acids compose cellular membranes, act as signaling molecules, and act as substrates for energy metabolism. Identifying which of these general cellular causes leads to disease represents an important first step in our understanding of neuronal requirements of lipids and will inform the efficient design of reliable diagnostics and therapies for ALD. Due to the known role of glucose and not fatty acids as the only energy substrate in the brain, which is almost completely nervous tissue, we speculated that degeneration in *ACSL* likely results from loss of a lipid-dependent signaling molecule or membrane component. Studies on phospholipase C suggest that in fact, these two cellular roles, at least in light-sensing photoreceptor cells, might represent a single defect (Hardie and Franze, 2012).

We initiated inquiry into whether *bgm* and *dbb* mutants shared a mechanism for neurodegeneration suggested by experiments using null mutants of *fatp*. Our hypothesis

of shared mechanism was founded in the shared function of both genes as ACSs as well as the shared neurodegenerative loss of function phenotype, and similar phenotypic dependencies on light exposure.

We concluded through these studies that Rh1 might be accumulated in *bgm* but not *dbb* mutants after approximately 8 days post-eclosion. Therefore, we cannot exclude the hypothesis that degeneration seen in *bgm* mutants is due to a defect in Rh1 signaling. However, further confirmation of this mechanism of neurodegeneration must be performed.

Retinal degeneration in null *fatp* mutants is cell autonomous, light dependent, and is reported to be rescued by a vitamin-A deficient diet. Vitamin A is the precursor for the rhosopdin chromophore and a vitamin A deficient diet rescues other retinal degeneration mutants in the genes *retinal degeneration B, crumbs*, and *arrestin (arr)* (Alloway et al., 2000; Harris and Stark, 1977; Johnson et al., 2002). The authors further support this notion by showing that over-expression of *rh1* in photoreceptors is sufficient for neurodegeneration. Molecularly, once rhodopsin receptors are activated, arrestin binds to an internal fragment and signals endocytosis of the activated receptor thereby quenching the excitatory signal and restoring the cell to a basal primed signaling state. Stabilization of an arrestin/rh1 complex leads to excitotoxic neurodegeneration (Chinchore et al., 2009; Kiselev et al., 2000; Satoh and Ready, 2005). To this point, *fatp; arr*^{2} retinas lost significantly less photoreceptors suggesting *fatp*$^{-/-}$ retinas degenerate due to an excitotoxic mechanism. Experiments further testing whether the mechanism of neurodegeneration is shared between *bgm* and *fatp* null mutants will be discussed in Chapter 5.

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Figure 4.1 *bgm* and *dbb* are expressed in the affected tissues of the adult retina. In situ hybridization of wild type and mutant animals with probes complementary to *bgm* (A) and *dbb* (B) transcript, respectively. *bgm* has a restricted staining that appears to be restricted to the basement membrane and around the medulla and possibly slight staining in the retina. *dbb* stains ubiquitously throughout the central brain and optic lobe in only wild type animals.

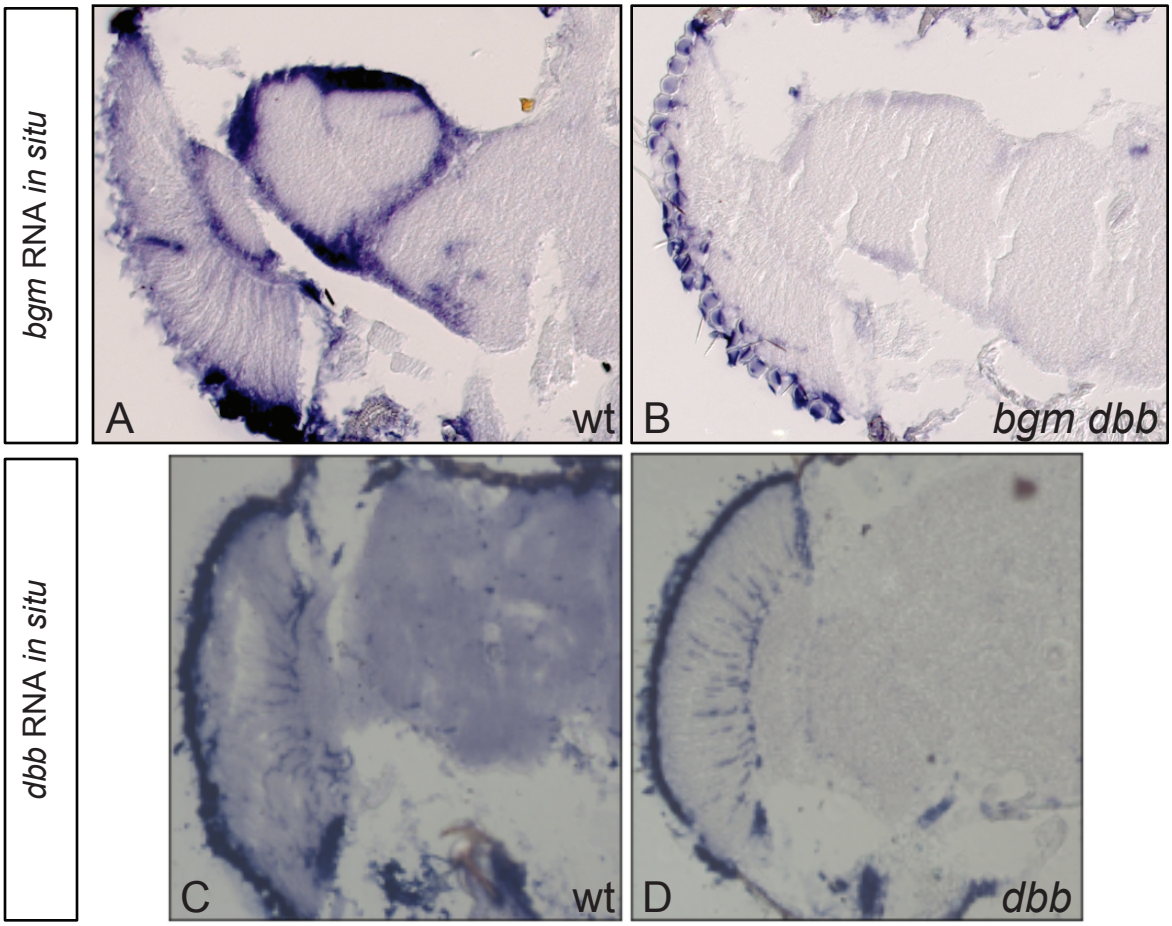
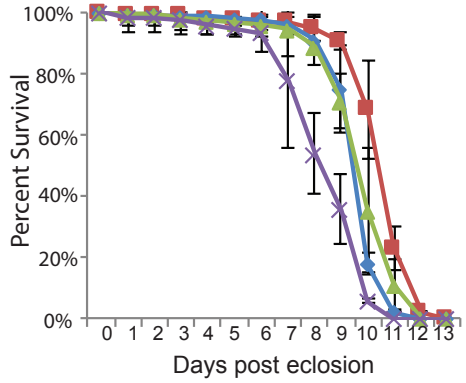
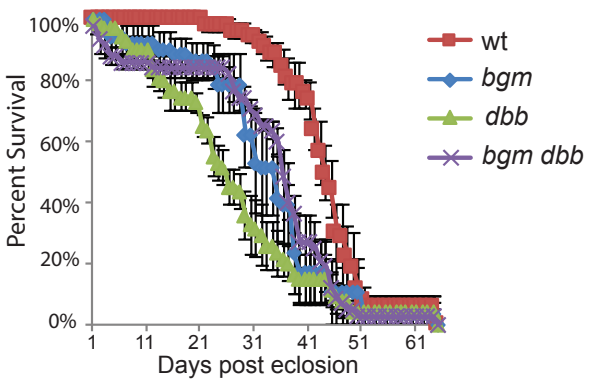

bgm RNA in situ
A
wt
B
bgm dbb
dbb RNA in situ
C
wt
D
dbb

Figure 4.2 Hyperoxia does not exacerbate lifespan defects in *bgm* and *dbb* animals. Death curves for animals raised from day 1 in hyperoxic conditions (A) compared to normoxic conditions (B). Error bars represent standard error of all biological replicates. Table showing the percent survival of each mutant normalized to the P50 of wild type in hyperoxia or normoxia shows no worsening lifespan when normalized to the hyperoxic effect on wild type animals. Shown are the results from three biological replicates. n=117-225/genotype

A



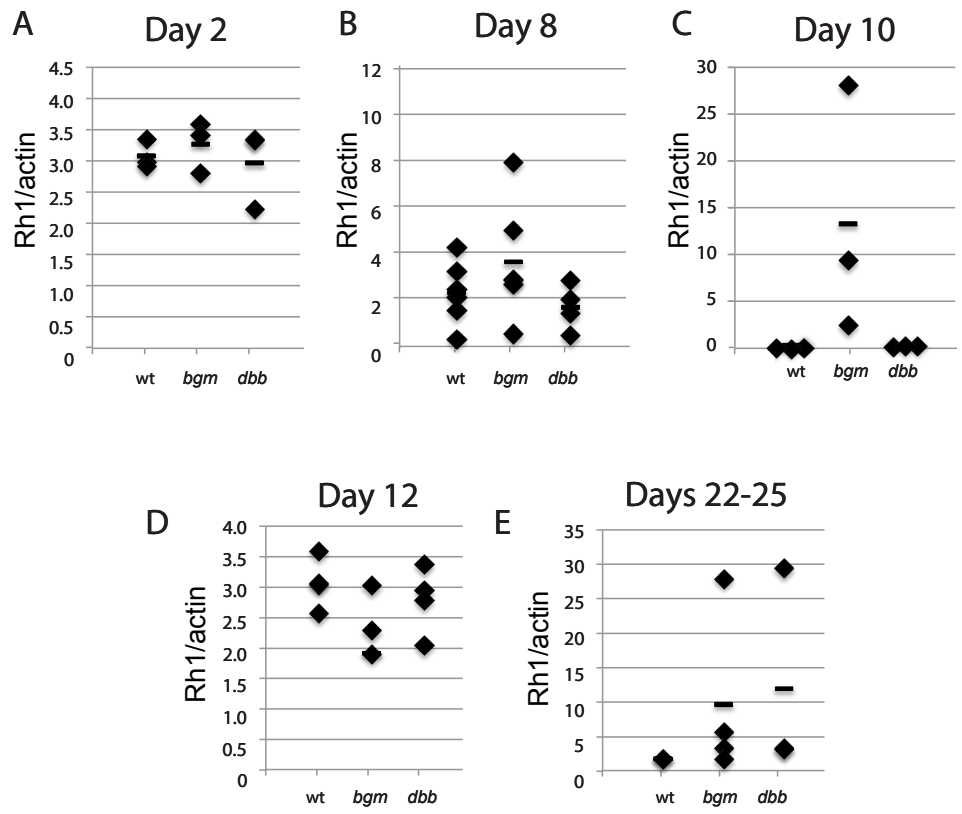
B



C

	Hyperoxic 50% survival	% of wt survival	Normoxic 50% survival	% of wt survival
wt	11 days	---	44 days	---
bgm	10 days	91%	35 days	80%
dbb	10 days	91%	26 days	59%
bgm dbb	9 days	82%	37 days	84%

Figure 4.3 Rh1 expression is highly variable, but some *bgm* mutant samples display higher levels of Rh1 than seen in wild type. Quantification of Rh1 monomer, dimer, and oligomer expression assayed by western blot in animals aged 2 (A), 8 (B), 10 (C), 12 (D), and 22-25 (E) days post-eclosion. Extensive variability is seen but some samples appear to contain higher than wild type levels of Rh1. Each animal is represented by a diamond while lines represent the average within each group. Representative images of Rh1 oligomer visualized with anti-Rh1 and actin (loading control) in young (dpe2) and old (dpe25) male animals (F). Red boxes indicate the area used for quantification using Odyssey software.


A
Day 2
Rh1/actin
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
wt
bgm
dbb
B
Day 8
Rh1/actin
12
10
8
6
4
2
0
wt
bgm
dbb
C
Day 10
Rh1/actin
30
25
20
15
10
5
0
wt
bgm
dbb
D
Day 12
Rh1/actin
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
wt
bgm
dbb
E
Days 22-25
Rh1/actin
35
30
25
20
15
10
5
0
wt
bgm
dbb
F
dpe2
dpe25
WT
bgm
dbb
WT
bgm
dbb
RH1
OLIGOMER
β-ACTIN

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

ALD is a fatal, childhood neurodegenerative disease most commonly caused by mutations in the *ABCD1* gene. Inheritance of the same null alleles in family members exhibit incomplete penetrance and variable expressivity of phenotypes—suggesting environmental and/or genetic modifiers while also complicating mechanistic studies of the disease. We have generated the only ALD animal model displaying the most severe aspect of ALD pathology—central nervous system degeneration. We utilized this model as a tool to probe the requirements of lipids in neuromaintenance and to answer long-standing questions regarding the mechanism of degeneration in ALD. We investigated the tissue-specific requirements of the ACSL pathway, as well as the mechanism of cell death in neurodegeneration.

We determined that Drosophila *bgm* is required in neuronal cells, and that the defects in neuromaintenance observed in *bgm* and *dbb* single mutants are due to a lack of terminally processed fatty acid product. We recapitulated the requirement of products derived from the ACSL pathway by driving RNAi-mediated knock down of a *Drosophila* elongase expressed in the adult retina and show a shared neurodegenerative defect that

also originates in neurons. Finally, we provide preliminary evidence that the terminally processed lipid product required in *bgm* and *dbb* animals plays a role in the rhodopsin-signaling cascade.

In this work, the most impactful finding translating to ALD is the suggestion that accumulating VLCFAs seen in both humans and *Drosophila bgm dbb* animals are not sufficient to cause disease and in fact, the lack of activated fatty acid product represents the true cause of cellular dysfunction. Evidence for the insufficiency of the toxic accumulation model was present in multiple forms in the literature; however, no study had parsed an accumulation of VLCFAs separately from a cellular lack of activated fatty acid product. As such, the hypothesis was tested here in multiple ways: 1) two diets, one designed to exacerbate the phenotype caused by an accumulation, and the other designed to provide an activated long/very long chain fatty acid product via another biochemical pathway; 2) environmental modulation of the requirement for product through rearing in darkness; and 3) restriction of the absolute amount of terminally activated product by blocking elongation of medium chain substrates using RNAi. Results from each of these experiments pointed to a model where "activated" long/very long chain fatty acids are required in neuromaintenance in adult *Drosophila*.

As mentioned, ALD is not unique in its disease etiology, being rooted in defects in lipid homeostasis as are other neurodegenerative diseases linked to genes whose encoded proteins play key roles in lipid metabolism (acid sphingomyelinase: Niemen Pick type A OMIM#257200, alpha-galatosidase A deficiency: Fabry disease OMIM#301500, and acid ceramidase: Farber's disease OMIM#228000). Although the three diseases referenced here are associated with an accumulation of lipid species

(sphingomyelin, globotriaosylceramide and ceramide, respectively), the mechanism of neuropathy is unknown (Ahmad et al., 2009; Madra and Sturley, 2010; Ries and Gal, 2006; Schiffmann, 2006; Vazquez et al., 2012; Zhang et al., 2000). Moreover, Fabry disease and Farber's disease both display variable expressivity suggesting as-yet-unidentified genetic and/or environmental modifiers, similar to ALD. At least for Farber's disease, intralysosomal ceramide accumulation is not correlated with cellular apoptosis in fibroblasts harboring a block in sphingolipid metabolism. Investigators hypothesize the cause of neurodegeneration is downstream of ceramidase function (Tohyama et al., 1999). The mechanism of disease is not likely shared between all of these lipid-mediated neurodegenerative diseases; however, that degeneration in our animal model of ALD is caused by a defect downstream of ACS function might provide some insight into mechanisms of lipid-mediated degeneration in several related conditions such as these.

ALD is also part of a larger family of leukodystrophies. As a whole, this family of diseases remains poorly understood and the identification of novel cellular mechanisms for diseases such as ALD can assist in our understanding of all diseases in this family. Only half of the patients who present in the clinic with a leukodystrophy can actually be traced to a cause. That a significant fraction of severely ill patients exist with no known cause underscores the need for increasing our basic understanding for the requirements of neuromaintenance. Altered lipid homeostasis in ALD manifests as a leukodystrophy. Therefore, mutations in other genes in this pathway may play a role in uncharacterized leukodystrophies as well. Further, these advances in the mechanistic understanding of neurometabolic disease are imperative to direct appropriate clinical therapies for ALD.

Use of the *bgm dbb* animal model also changes our perception of how current therapies for ALD work. For example, either allogenic hematopoietic cell transplant (HCT) or autologous transplant with correction of the *ABCD1* gene can halt neurodegeneration (Baumann et al., 2003; Cartier et al., 2009). Although not yet clear why, it is thought that transplanted microglial cells express *ABCD1*, thereby replacing dysfunctional microglia and rescuing degeneration. However, our *bgm* rescue experiment (Chapter 3) suggests the ACSL pathway is required in neuronal cells and not glial cells, thereby opening the possibility that HCT transplant halts neurodegeneration because it eliminates the ability of the immune system to rapidly exacerbate demyelination. This suggests that a strong dampening of the immune system would be as effective as an immune transplant in patients (although the earlier would leave the patient in a vulnerable, nonideal immune-suppressed state). Additionally, this suggests that autologous transplantations with lentivirally-transduced HCT to express wild-type *ABCD1* is an unnecessary step that results in an outcome no better than immune suppression or allogenic transplants. Indeed, autologous transplants have not yet been shown to be more effective than allogenic transplants (Cartier et al., 2012).

The need for the biological lessons surrounding lipid requirements of the nervous system is dire. We have used the *bgm* and *dbb Drosophila* model of ALD to address key questions not previously considered: regarding the tissue specific requirements of long and very long chain fatty acids and the cellular mechanisms of degeneration resulting from deficiencies in this pathway. As is the beauty of scientific research, these studies have led us not only to answers, but to more questions as well. It seems likely that the full utility of our *bgm dbb* model of ALD has yet to be fully realized.

Future Directions

Subcellular pathway requiring terminally processed

long/very long chain fatty acid

Dourlan and colleagues have suggested that the cytotoxic phenotype seen in mutants null for *fatp* is dependent on Rhodospin activation (Dourlen et al., 2012). Since Rhodopsin synthesis can be halted with a diet deficient in vitamin A, it will be important to determine if this diet can rescue *bgm* and *dbb* mutant animals as it does animals with the null $fatp^{k10307}$ allele. Additionally, if degeneration in *bgm* and *dbb* animals is due to cytotoxic activation of photoreceptor cells leading to autonomous cell death as it is for animals with null alleles of *fatp*, a *bgm* arr^{1} or *dbb* arr^{1} mutant should rescue degeneration in these animals (alternatively the dominant negative allele of rhodopsin—$rh1^{G69D}$—can be used for double mutant analysis). Determining if *bgm* and *dbb* mutant degeneration is due to an excitotoxic mechanism dually informs the mechanism of neurodegeneration in *bgm* and *dbb* mutants (excitotoxicity) as well as *fatp* mutants (likely due to ACS function).

Candidate Interacting Genes and Double Mutant Analysis

Seizure or trauma

To test the hypothesis that stress by use or by trauma can exacerbate neurodegeneration in tissues with defects in ACSL metabolism, Drosophila seizure-sensitive mutants can be utilized. I have generated four lines that carry the bang seizure-sensitive allele of *paralytic* on the X chromosome and are either WT, or homozygous for *bgm, dbb*, or *bgm dbb* null alleles on the second chromosome. $para^{bss1}$ is a gain-of-

function allele resulting from a missense mutation in the "paddle motif" which has been implicated in inactivation of the voltage-gated sodium channel Paralytic protein product (Parker et al., 2011). Hemizygous *para*^{bss1}/Y males experience initial seizure, paralysis, and clonus-like seizures unique to *bss1* alleles followed by a recovery seizure and recovery. To test whether seizures represent an environmental (or sometimes genetic in the case of our PRRT2 allele in Chapter 2) modifier of the ACSL-deficient neurodegeneration, a daily seizures regime from day 1 post-eclosion to day 20 post eclosion in A) *para*^{bss1}/Y males should be compared to B) *para*^{bss1}*; bgm*^{1} C) *para*^{bss1}*; dbb*^{1} D) *para*^{bss1}*; bgm*^{1} *dbb*^{1} animals under the same regime.

Alternatively, a recent publication describes a Drosophila model of traumatic brain injury that can be used to confirm results with the *para*^{bss1} experiments or to stand alone as support for the hypothesis that trauma can exacerbate neurodegeneration in *bgm* and *dbb* mutants (Katzenberger et al., 2013).

ABCD1 and *bgm*, *dbb* in the ACSL pathway

It is of obvious interest to determine if *bgm* and *dbb* mutants reside in the same biochemical pathway as *dABCD1* mutants. There are no described genetic mutants of *dABCD1,* although there are a few possibilities located in large-scale screens (alleles *MI11268, f00836, 39C-34*, and *5-HA-1572* in FlyBase). However, observing an aged *bgm*; *elav>ABCD1*-RNAi (or the equivalent *dbb*, or *bgm dbb* animal) and comparing severity of neurodegeneration to the already-characterized genetic single mutants or an animal with only *elav>ABCD1*-RNAi would likely produce results that suggest ACSs and the ABCD1 transporter reside in the same linear pathway. I would predict no worsening

of the phenotype in animals with disrupted *dABCD1* and ACS function.

Elongase- *CG2781*

Similar to *ABCD1*, it would be of interest to determine if the *bgm* and *dbb* ACSs and elongases are, in fact, parallel pathways. Similar to dABCD1, no genetic null alleles of *CG2781* have been reported but there are a few p-element insertions that may disrupt gene function (alleles: *c06208* and *MI01455*). My model would suggest a retinal phenotype exists if these alleles are genetic null alleles. If a retinal phenotype is identified and can be exacerbated *ACS, CG2781* double mutant animals could be examined. If no genetic mutant can be used *ACS*; neuronal-GAL4>*CG2781-RNAi* can be examined. In contrast to my prediction for the genetic interaction of *bgm* and *dbb* with *dABCD1*, I would postulate that *ACS, CG2781* mutants (either genetic or elongase-RNAi) would have a worse phenotype than either of the single mutants suggesting these represent parallel pathways to generate the activated long/very long chain lipid product. Of note, CG2781 has been mentioned once in the literature as a gene that is upregulated in null mutants of the neuronally expressed transcription factor gene *erect wing* (Haussmann et al., 2008). No further characterization was performed.

One alternative to this experiment is the analysis using an allele of the only identified trans-enoyl reductase, *Sc2*, which encodes the terminal step of the four-step fatty acid elongation. Two alleles of this gene exist: $Sc2^{05634}$ and $Sc2^{EY02236}$. $Sc2^{05634}$ is a putative null allele exhibiting larval lethality. Therefore, clonal analysis in a *bgm* and *dbb* mutant background will be required to observe retinal clones of *bgm;*$Sc2^{05634}$ ommatidia in adult flies. I predict that given Sc2 catalyzes the terminal step to fatty acid elongation

and my data suggest input from both elongation and activation pathways are required for neuromaintenance, double mutant retinal clones will display a worse phenotype than *ACS* or *Sc2* single mutants (Chen et al., 2010; Gambis et al., 2011). The *EY02236* allele is the result of a UAS element insertion at the endogenous locus and on its own results in a weak loss-of-function, but upon induction can be over expressed. An alternative approach would be to determine if Sc2 expression is required in a *bgm* and *dbb* mutant background for animals to be rescued on the medium chain diet. A medium chain diet ameliorated retinal degeneration in *bgm* and *dbb* animals through an abundance of substrate for the elongase pathway. I would hypothesize that expression of *Sc2* is required for *bgm* and *dbb* mutant rescue on a medium chain diet and therefore disruption of Sc2 function with either allele in an ACS mutant background will prevent rescue.

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